MDM2 Binding Induces a Conformational Change in p53 That Is Opposed by Heat-shock Protein 90 and Precedes p53 Proteasomal Degradation* 

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p53 protein conformation is an important determinant of its localization and activity. Changes in p53 conformation can be monitored by reactivity with wild-type conformation-specific (pAb-1620) or mutant conformation-specific (pAb-240) p53 antibodies. Wild-type p53 accumulated in a mutant (pAb-240 reactive) form when its proteasome-dependent degradation was blocked during recovery from stress treatment and in cells co-expressing p53 and MDM2. This suggests that conformational change precedes wild-type p53 degradation by the proteasome. MDM2 binding to the p53 N terminus could induce a conformational change in wild-type p53. Interestingly, this conformational change was opposed by heat-shock protein 90 and did not require the MDM2 RING-finger domain and p53 ubiquitination. Finally, ubiquitinated p53 accumulated in a pAb-240 reactive form when p53 degradation was blocked by proteasome inhibition, and a p53-ubiquitin fusion protein displayed a mutant-only conformation in MDM2-null cells. These results support a model in which MDM2 binding induces a conformational change that is opposed by heat-shock protein 90 and precedes p53 ubiquitination. The covalent attachment of ubiquitin may "lock" p53 in a mutant conformation in the absence of MDM2-binding and prior to its degradation by the proteasome.

Inactivation of p53 is considered essential for the development of most or all human cancers (1). More than 50% of human cancers harbor inactivating mutations in the p53 gene, and in cancers that retain wild-type p53, other defects in the p53 tumor suppressor pathway are observed. Cancer-associated p53 mutations are found almost exclusively in the DNA binding domain and inhibit the ability of p53 to activate expression of its downstream target genes (2, 3). These cancer-associated mutations also alter p53 protein conformation to varying extents. Changes in p53 conformation can be assessed by monitoring reactivity with conformation-specific antibodies that recognize p53 in either a wild-type (pAb-1620) or mutant (pAb-240) conformation (4, 5). Wild-type p53 is a flexible and conformationally labile protein, and conditions that can alter its conformation have been examined. For example, Milner and Watson (6) reported that wild-type p53 switched to a mutant conformation in serum-stimulated murine fibroblasts. In addition, Milner and Medcalf (7) demonstrated that formation of hetero-oligomers of wild-type and mutant p53 proteins could drive the wild-type protein into a mutant conformation. Regulated changes in p53 protein conformation are likely to be important determinants of p53 activity.

Heat-shock protein 90 (Hsp90) is a molecular chaperone that plays an essential role in the conformational maturation of numerous proteins, including nuclear receptors, transcription factors, and protein kinases (8). It is believed that Hsp90 maintains these proteins in an active conformation that can be rapidly triggered after stimulus. Hsp90 also functions in the folding of newly synthesized proteins and their refolding after conditions of denaturing stress. Functions of Hsp90 may be modulated by association with co-chaperones such as Hsc70, Hsp40, and Hop (8, 9). Complexes between Hsp90 and wild-type p53 have been recognized, and it is currently believed that Hsp90 is a positive regulator of p53. For example, the Hsp90 inhibitor geldanamycin (GA) diminished the stress-induced accumulation and activation of p53 in a camptothecin-treated human fibroblast cell line (10) and in cells exposed to heat shock (11). Hsp90 inhibition was also reported to block the nuclear accumulation and reactivation of p53 when cells harboring a temperature-sensitive p53 mutant were shifted to the permissive temperature (12, 13). And finally, purified Hsp90 protein maintained p53 in a wild-type, DNA binding conformation at elevated temperatures in vitro (10). Together, these results support a role for Hsp90 in maintaining the levels, activity, and conformation of wild-type p53.

MDM2 is the product of a p53-inducible gene and can bind the N terminus of p53 and inhibit its transcriptional activity (14, 15). Importantly, MDM2 binding can also promote the ubiquitination of p53 and its degradation by the proteasome (16, 17). This may allow a more efficient inhibition of p53 activity than would result from MDM2 binding alone. Current models suggest MDM2 is an E3 ubiquitin-protein ligase that facilitates ubiquitin transfer to p53 (18). Various reports have suggested a

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14626 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 282 • NUMBER 19 • MAY 11, 2007

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possible relationship between the conformation of p53 and its degradation by the proteasome. For example, El-Deiry and coworkers (19) reported that the drug CP-31398 could stabilize endogenous p53 in cultured cells. CP-31398 can maintain p53 in an active conformation and can drive some mutant p53s into a wild-type conformation (20). The fact that it could block p53 degradation raised the possibility that wild-type p53 might assume a mutant conformation before its degradation. The plant alkaloid ellipticine can also maintain p53 in a wild-type conformation and can drive some mutant p53s into a wild-type conformation (21). Like CP-31398, ellipticine treatment stabilized endogenous wild-type p53 in cultured cells (21, 22), supporting the possibility that wild-type p53 might assume a mutant conformation before degradation. In the current study we monitored p53 protein conformation under conditions in which its MDM2 and proteasome-dependent degradation was inhibited. Our results support a model in which MDM2 binding promotes a conformational change in p53 that is opposed by the addition of empty plasmid. Where indicated, the proteasome inhibitor MG132 (Boston Biochem) was added to a final concentration of 30 μM.

Conformational Change Precedes p53 Proteasomal Degradation

EXPERIMENTAL PROCEDURES

Plasmid DNAs—DNA encoding p53 Δ42N has been described (23) and was from Peter Howley (Harvard Medical School). FLAG-tagged wild-type p53 has been described (24) and was from Zhimin Yuan (Harvard School of Public Health). This DNA contains wild-type p53 sequences cloned into BamHI and XbaI sites downstream of the FLAG epitope. DNAs encoding the untagged p53 and the p53-ubiquitin fusion protein were from Wei Gu (Columbia University) (25). Myc-tagged ubiquitin DNA was from Ron Kopito (Stanford University) (26). MDM2 Δp53BD lacks residues 52–96 in the MDM2 N terminus. DNA encoding wild-type MDM2 and MDM2 Δp53BD were from Steve Grossman (University of Massachusetts Medical School). MDM2 ΔRING DNA encodes residues 6–339 (27). This DNA was from Arnold Levine. DNA encoding FLAG-tagged Hsp90 was provided by Len Neckers (NCI, National Institutes of Health).

Cell Culture and Transfections—MCF7 (breast cancer), U2OS (osteosarcoma), and HepG2 (liver cancer) cell lines all express wild-type p53. MDM2/p53 double knock-out mouse embryo fibroblasts were from Rudy Alarcon (Stanford University). All cell lines were maintained at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics (1% penicillin and streptomycin). Control siRNA or siRNA against MDM2 (smart-pool) were from Dharmacon and were transfected to Hep3B cells using Dharmafect reagent, also from Dharmacon. Transfections in U2OS or MDM2/p53 double knock-out cells were done using FuGENE-6 transfection reagent (Roche Applied Science) according to the manufacturer’s protocol when cells were ~60% confluent. Total DNA in each transfection was equalized by the addition of empty plasmid. Where indicated, the proteasome inhibitor MG132 (Boston Biochem) was added to a final concentration of 30 μM 18 h after transfection, and the cells were incubated for an additional 5–7 h before harvesting. MCF7 cells were treated with Hsp90 inhibitors at the following final concentrations: 17-AAG (10 μM), GA (10 μM), radicicol (0.5 μM). 17-AAG was from Ralph Weichselbaum (University of Chicago), GA was from A. G. Scientific, and radicicol was from Sigma.

Immunoprecipitations and Immunoblotting—To harvest cell lysates for immunoblotting, cells were rinsed with 2 ml of phosphate-buffered saline and then scraped into 700 ml of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, phenylmethylsulfonyl fluoride, leupeptin) and transferred to microcentrifuge tubes. The cells were then incubated on ice for 30 min with occasional vortexing and spun at 4 °C, 14,000 rpm for 15 min to remove cellular debris. For immunoprecipitations using conformation-specific p53 antibodies, cell lysates were first mixed for 1 h with 30 μl of protein A-agarose beads to pre-clear the lysate. The lysate was then divided into equal halves, and each half was immunoprecipitated overnight with 1.75 μl of either the wild-type conformation-specific (pAb-1620, Invitrogen Ab-5) or mutant conformation-specific (pAb-240, Invitrogen Ab-3). Immunoprecipitates were captured by incubation with 30 μl of protein A-agarose beads for 1 h, and the beads were isolated by centrifugation for 10 s at 13,000 rpm. The beads were then washed twice with 1 ml of ice-cold lysis buffer, boiled for 10 min, and then resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes for immunoblotting. Antibodies used in immunoblotting to detect p53 included the sheep polyclonal antibody Ab-7 (Oncogene Science), monoclonal p53 antibody 1801 (Oncogene Science), and the FLAG monoclonal antibody Ab-5 (Sigma-Aldrich). MDM2 antibody was SMP-14 (Santa Cruz). Tubulin antibody Ab-1 was from Santa Cruz.

RESULTS

p53 Accumulates in a Mutant Conformation When Its Proteasome and MDM2-dependent Degradation Is Inhibited—We wished to test whether endogenous p53 undergoes a conformational change before degradation by the proteasome. Wild-type p53 is stabilized and its levels increase in cells after DNA-damaging stress, and cells undergo G1- or G2–phase cell cycle arrests and DNA repair (28). p53 is subsequently degraded, and its levels decrease when DNA repair is complete and cells resume normal cell cycling (29, 30). p53 degradation during stress recovery is believed to be MDM2-dependent (29, 30). In preliminary experiments we first monitored p53 levels in MCF7 cells exposed to actinomycin D (ActD) for 4 h and then allowed them to recover from ActD treatment for various time periods (Fig. 1A). p53 levels were increased after 4 h of ActD treatment, remained elevated for 7 h after removal of ActD, and subsequently decreased at the 10, 12, 16, and 20 h time points. These results suggest p53 degradation resumes between 7 and 10 h after ActD removal. MDM2 levels were also increased in cells exposed to ActD for 4 h and remained elevated at all time points after removal of ActD with a peak at 7 h. Next, we tested whether the decrease in p53 levels after ActD removal could be blocked by proteasome inhibition. As shown in Fig. 1B, p53 levels were increased by ActD treatment for 3 h, remained elevated 6 h after ActD removal, and were decreased 12 h after ActD removal. In contrast, the decrease in p53 levels between 6–12 h after ActD removal was blocked in cells treated with the proteasome inhibitor MG132 (compare p53 levels in lanes 4

MAY 11, 2007•VOLUME 282•NUMBER 19 JOURNAL OF BIOLOGICAL CHEMISTRY 14627
Conformational Change Precedes p53 Proteasomal Degradation

**FIGURE 1.** Wild-type p53 accumulates in a mutant conformation after proteasome inhibition. A, MCF7 cells (p53 wild-type) were untreated or exposed to 30 ng/ml ActD for 4 h. The cells were then rinsed twice with phosphate-buffered saline and cultured for the indicated times in the absence of ActD (remove ActD). p53 and MDM2 levels were assessed. B, MCF7 cells were untreated (nt) or exposed to 30 ng/ml ActD for 3 h followed by removal of ActD and continued culture for 6 or 12 h. In some cases (lane 5) cells were cultured for 6 h in the absence of ActD followed by an additional 6 h in the presence of proteasome inhibitor MG132 (30 μM final concentration). p53 and MDM2 levels were assessed. p53 levels decreased between 6 and 12 h after ActD removal in the absence of MG132, and this decrease was blocked by proteasome inhibition. C, lysates from lanes 2–5 in B were immunoprecipitated with wild-type conformation-specific (pAb1620) or mutant conformation-specific (W, pAb240) antibodies and examined by immunoblotting (IB) with the p53 polyclonal antibody Ab-7 and the MDM2 antibody SMP-14.

**FIGURE 2.** p53 accumulates in a mutant conformation when its MDM2-dependent degradation is blocked by proteasome inhibition. A, U2OS cells were transfected with DNA encoding FLAG p53 (100 ng) either alone or with MDM2 (1 μg). Where indicated cells were treated with MG132 (30 μM final concentration, 5.5 h treatment) 17 h after transfection. Transfected cell lysates were examined by immunoblotting with anti-FLAG antibody heavy used in the immunoprecipitation. The upper band indicated by the asterisk is detection of the antibody heavy used in the immunoprecipitation. The lower band indicated by the arrow is FLAG p53.

and 5, Fig. 1B). This indicates the decrease in p53 levels between 6 and 12 h after ActD removal is proteasome-dependent. Finally, we monitored p53 conformation in response to ActD treatment and during recovery in which it was undergoing proteasomal degradation. Treated cell lysates were immunoprecipitated with wild-type conformation-specific (pAb1620) or mutant conformation-specific (pAb240) p53 antibodies followed by immunoblotting with the p53 antibody Ab-7. p53 immunoprecipitated by each antibody in these experiments is an indicator of p53 conformation. As shown in Fig. 1C, p53 displayed a mostly wild-type conformation (recognized by the wild-type conformation-specific antibody but not the mutant conformation-specific antibody) in cells treated with ActD for 3 h and in cells 6 h after ActD removal. At the 12-h time point after ActD removal, when p53 levels were decreased, p53 appeared to be evenly distributed between a wild-type and mutant conformation. Strikingly, however, p53 accumulated in a mutant (pAb240-reactive) conformation when its proteasomal degradation between 6 and 12 h after ActD removal was blocked by MG132 treatment, and abundant MDM2 binding to p53 was also observed (Fig. 1C). This suggests p53 undergoing proteasomal degradation during recovery from stress treatment may assume a mutant conformation before degradation.

Wild-type p53 undergoes proteasome-dependent degradation when co-expressed with excess MDM2. We monitored p53 conformation under conditions in which it was being targeted for degradation by MDM2 in co-transfected cells. U2OS cells were transfected with DNAs encoding FLAG-tagged p53 either alone or with excess MDM2. In some cases cells were treated with MG132 to inhibit the proteasome before harvesting, and p53 and MDM2 levels were assessed. As expected, p53 levels decreased with MDM2 co-expression, and this decrease was blocked by proteasome inhibitor treatment (Fig. 2A). This is consistent with MDM2 promoting p53 degradation in a proteasome-dependent manner. Next, we monitored p53 conformation in the transfected cell lysates. As shown in Fig. 2B, FLAG p53 maintained a mostly wild-type conformation when expressed alone and, whereas p53 levels decreased with co-expression of MDM2, there appeared to be a slight shift toward the mutant conformation. Importantly, blocking p53 degradation by proteasome inhibition caused an increase in mutant conformation p53 and decrease in wild-type conformation p53 (Fig. 2B), suggesting a shift from wild-type to mutant conformation before p53 degradation.

Ubiquitinated p53 and a p53-Ubiquitin Fusion Protein Have a Mostly Mutant Conformation—MDM2 is an E3-ligase that can ubiquitinate p53 to promote its degradation. This led us to...
Conformational Change Precedes p53 Proteasomal Degradation

FIGURE 3. Ubiquitinated p53 accumulates in a mutant conformation after proteasome inhibition. A, U2OS cells were transfected with DNAs encoding FLAG p53 (100 ng), MDM2 (1 mg), and Myc-Ub (200 ng) as indicated. In some cases cells were treated with MG132 (30 μM final concentration, 5.5 h treatment) 17 h after transfection. FLAG p53 and MDM2 levels are shown. Tubulin is a loading control. B, transfected cell lysates were immunoprecipitated with wild-type conformation-specific (W, pAb1620) or mutant conformation-specific (M, pAb240) antibodies followed by immunoblotting with an anti-FLAG antibody or anti-MDM2 antibody. The lower band indicated by the asterisk is detection of the antibody heavy used in the immunoprecipitation. The upper band indicated by the arrow is FLAG p53. Shorter exposure of the FLAG blot (middle panel) shows conformation of the non-ubiquitinated p53 in each condition. Longer exposure (upper panel) allows detection of ubiquitinated p53.

FIGURE 4. p53 ubiquitin fusion protein has a mutant conformation. MDM2/p53 double-knock-out mouse embryo fibroblasts were transfected in duplicate with DNA encoding untagged wild-type p53 or a p53-ubiquitin (p53-Ub) fusion protein. Transfected cell lysates were immunoprecipitated with wild-type conformation-specific (W, pAb1620) or mutant conformation-specific (M, pAb240) antibodies, followed by immunoblotting with the anti-p53 polyclonal antibody Ab-7. The bands indicated by the arrows are full-length wild-type p53 and the p53-Ub fusion protein. The low molecular weight bands below the full-length proteins may represent an internal translation product of p53.

We noted that when p53 is stabilized by proteasome inhibition, it accumulates mostly as a full-length band that is not obviously modified (not ubiquitinated). This led us to suspect that conformational change in p53 may precede its ubiquitination by MDM2. Efficient p53 degradation requires MDM2 input examine the conformation of wild-type p53 that is ubiquitinated by MDM2. To this end, U2OS cells were transfected with DNAs encoding FLAG p53, MDM2, and Myc-tagged ubiquitin (Myc-Ub), in different combinations (Fig. 3A). Myc-Ub was included in these experiments to facilitate detection of ubiquitinated p53 species. In some cases transfected cells were treated with MG132 to inhibit the proteasome before analysis. As shown in Fig. 3A, p53 levels decreased when co-expressed with MDM2 alone or MDM2 plus Myc-Ub, and this decrease was blocked by MG132. This is consistent with MDM2 promoting p53 degradation by the proteasome. Transfected cell lysates were then immunoprecipitated with wild-type (pAb1620) or mutant (pAb240) conformation-specific p53 antibodies and examined by immunoblotting with anti-FLAG antibody. FLAG p53 maintained a mostly wild-type conformation when expressed alone (Fig. 3B, middle panel). When p53 was co-expressed with excess MDM2 alone or MDM2 plus Myc-Ub, the p53 that remained appeared to be shifted slightly toward a more mutant conformation (Fig. 3B, middle panel). Consistent with Fig. 2, p53 again accumulated in a mutant conformation when its degradation by MDM2 alone or MDM2 plus Myc-Ub was blocked by proteasome inhibitor treatment (+ MG132, Fig. 3B, middle panel). Long exposures of the film in Fig. 3B, middle panel, were used to assess conformation of ubiquitinated p53 in higher molecular weight, p53-ubiquitin conjugates. In each case where ubiquitinated p53 could be observed, it displayed a mutant or mostly mutant conformation (Fig. 3B, upper panel). We noted in some, but not all experiments that ubiquitinated p53 levels were somewhat decreased with MG132 treatment (as example, compare ubiquitinated p53 levels with MDM2 and Myc-Ub alone versus MDM2, Myc-Ub, and MG132, Fig. 3B). This is consistent with reports that endogenous ubiquitinated p53 levels decreased in MG132-treated cells (31). To examine the conformation of ubiquitinated p53 further, we made use of a p53-ubiquitin fusion protein (p53-Ub) believed to mimic mono-ubiquitination of the p53 C terminus (25). p53/MDM2 double-null cells were transfected with DNAs encoding wild-type p53 or p53-Ub, and conformation of the expressed proteins was monitored. As shown in Fig. 4, wild-type p53 had a mostly wild-type conformation, whereas the p53-Ub fusion protein had a completely mutant (pAb240-reactive) conformation. These results were confirmed in duplicate experiments in Fig. 4 and support the idea that ubiquitinated wild-type p53 has a mutant conformation.

MDM2 Binding Promotes a Conformational Change in p53—
Conformational Change Precedes p53 Proteasomal Degradation

amounts be in excess to p53 (25). To test if MDM2 binding can affect p53 conformation, cells were transfected with equal amounts (1 μg of each) DNA encoding FLAG-tagged p53 and MDM2. p53 is not efficiently degraded by MDM2 under these conditions (25), and we could, therefore, monitor its conformation while maintaining protein levels. p53 conformation was then monitored by IP with the conformation-specific antibodies followed by probing with anti-FLAG antibody. As shown in Fig. 5B, p53 wild type (wt) had a mostly wild-type conformation when expressed alone but shifted toward a mutant conformation when either co-expressed with MDM2 or in cells treated with GA. Notably, a pronounced accumulation of p53 in a mutant conformation was observed when cells co-expressing p53 and MDM2 were treated with GA, and this coincided with decreased p53 in a wild-type conformation (Fig. 6A). Similar results were obtained in cells treated with GA. As shown in Figs. 6, B and C, FLAG p53 again had a mostly wild-type conformation when expressed alone but shifted toward mutant conformation when either expressed with MDM2 or in cells treated with GA (evidenced by accumulation of p53 recognized by the mutant (M) conformation-specific antibody). In contrast, a pronounced shift toward mutant conformation was observed when cells expressing p53 and MDM2 were treated with GA (Fig. 6B, MDM2 + GA), coincident with decreased p53 in a wild-type conformation. Finally, radicicol alone (0.5 μM) had little or no effect on p53 conformation (Fig. 6D). However, a pronounced shift toward mutant conformation was observed when cells expressing p53 and MDM2 were treated with radicicol. Thus, MDM2 expression combined with Hsp90 inhibition causes a pronounced p53 conformational change.

We next tested whether similar effects could be seen with MDM2 ∆RING that cannot ubiquitinate p53. As shown in Fig. 7A, there was accumulation of p53 in a mutant conformation when expressed with MDM2 ∆RING, consistent with results from Fig. 5. However, p53 was markedly shifted toward a mutant conformation when expressed with MDM2 ∆RING and treated with GA. Thus, the pronounced conformational change observed when cells expressing p53 and MDM2 were treated with Hsp90 inhibitor (GA) did not require the MDM2 ∆RING domain. To test if MDM2 binding to p53 is required, we first monitored conformation of p53 ∆42N, which lacks the N-terminal MDM2-binding domain. As shown in Fig. 7A, p53 ∆42N maintained a wild-type conformation when expressed alone or with MDM2 ∆RING and remained mostly wild type with only a slight shift toward the mutant conformation when expressed with MDM2 ∆RING and treated with GA.

Overall, these results suggest that MDM2 binding can promote a p53 conformational change, evidenced by exposure of the mutant (pAb240) epitope.

Hsp90 Inhibition and MDM2 Cause Pronounced p53 Conformational Change—We noted that a significant portion of p53 remained in a wild-type conformation when expressed with MDM2, apparently resistant to conformational changes that might be caused by MDM2 binding. Based on this, we speculated that one or more factors might associate with wild-type p53 and oppose conformational changes caused by MDM2. Hsp90 seemed a likely candidate since it has been reported to maintain wild-type p53 conformation and activity (10, 12). To examine the effects of MDM2 and Hsp90 on p53 conformation, and we made use of three different Hsp90 inhibitor compounds; the ansamycin antibiotic GA, 17-AAG (a derivative of GA), and radicicol. Radicicol is unrelated structurally to either GA or 17-AAG. Cells transfected with FLAG-tagged p53 and MDM2 DNA (1 μg each) were either untreated or treated with GA, 17-AAG, or radicicol to inhibit endogenous Hsp90 protein, and p53 conformation was assessed. As shown in Fig. 6A, FLAG p53 had a mostly wild-type conformation when expressed alone but shifted toward a mutant conformation when either co-expressed with MDM2 or in cells treated with 17-AAG. Notably, a pronounced accumulation of p53 in a mutant conformation was observed when cells co-expressing p53 and MDM2 were treated with 17-AAG, and this coincided with decreased p53 in a wild-type conformation (Fig. 6A).

FIGURE 5. MDM2 binding exposes the mutant (pAb-240) epitope in wild-type p53. U2OS cells were transfected with DNA encoding FLAG p53 wt or FLAG p53 ∆42N either alone or with DNA encoding MDM2 that was wt or lacked the RING-finger domain that is required for ubiquitination activity (∆RING). A, immunoblotting shows the levels of FLAG p53 and MDM2 proteins. Tubulin levels were used as a loading control. B, transfected cell lysates were immunoprecipitated (IP) with wild-type conformation-specific (W, pAb1620) or mutant conformation-specific (M, pAb240) antibodies followed by immunoblotting (IB) with anti-FLAG antibody and anti-MDM2 antibody.

MDM2 RING also caused an accumulation of p53 in a mutant conformation. As shown in Fig. 5B, FLAG p53 again had a mostly wild-type conformation when expressed alone but shifted toward a mutant conformation when either co-expressed with MDM2 or in cells treated with GA. As shown in Figs. 6A, B and C, FLAG p53 with expression of MDM2 wt (levels Fig. 5A). This indicates accumulation of mutant conformation p53 is not due to an overall increase in p53 levels. These results suggest MDM2 binding can promote a p53 conformational change, evidenced by exposure of the mutant (pAb240) epitope.

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Conformational Change Precedes p53 Proteasomal Degradation

Thus, the change in p53 conformation is largely dependent on p53 N terminus, consistent with the notion that MDM2 must bind the p53 N terminus to drive wild-type p53 into a mutant conformation in Hsp90 inhibitor-treated cells. To examine this further, we monitored wt p53 conformation when expressed with a MDM2 mutant that lacks the p53 binding domain (MDM2 Δp53BD). As shown in Fig. 7A, wt p53 maintained a wild-type (or mostly wild-type) conformation when expressed with MDM2 Δp53BD and either untreated or treated with GA, consistent with MDM2-p53 binding being required to drive p53 into a mutant conformation in GA-treated cells. Finally, we tested whether Hsp90 overexpression could diminish the change in p53 conformation observed when cells expressing p53 and MDM2 are treated with GA. As shown in Fig. 7B, wild-type p53 maintained a mostly wild-type conformation when expressed alone or with Hsp90. Consistent with the results in Fig. 7A, p53 switched to a mutant conformation when expressed with MDM2 A\text{RING} and treated with GA. Importantly, this effect was partially blocked in cells overexpressing Hsp90 (compare MDM2 A\text{RING} + GA with MDM2 A\text{RING} + GA + Hsp90, Fig. 7B). This result is consistent with GA affecting p53 conformation through inhibition of Hsp90. Together, the results of Figs. 5–7 indicate that MDM2 binding to the p53 N terminus can drive p53 into a mutant conformation, evidenced by exposure of the mutant pAb240 epitope. This effect is opposed by Hsp90 and does not require the MDM2 RING finger domain and p53 ubiquitination.

Hsp90 Inhibition Promotes Proteasome and MDM2-dependent Degradation of Wild-type p53—We reasoned that if conformational change is important for p53 degradation, then Hsp90 would diminish p53 degradation by MDM2, whereas HSP90 inhibitors would enhance p53 degradation by MDM2. To examine these possibilities, we first monitored p53 degradation in cells transfected with FLAG-p53 and increasing amounts of MDM2 either alone or with co-transfected Hsp90 (Fig. 8). p53 decreased with increasing amounts MDM2, and this was diminished by Hsp90 coexpression. Next, endogenous p53 levels were monitored in MCF7 cells (wild-type p53) treated with GA or 17-AAG alone or co-tREATED with proteasome inhibitor (MG132). As shown in Fig. 9A, p53 levels decreased after GA and 17-AAG treatment, and this was blocked by proteasome inhibition. To address whether the decrease in p53 was MDM2-dependent, we made use of nutlin, a compound designed specifically to occupy the p53 binding pocket in MDM2 and, thus, block p53-DMMD2 binding. As shown in Fig. 9A, co-treatment with nutlin blocked the p53 decrease caused by Hsp90 inhibitors GA and 17-AAG. Finally, we monitored p53 levels after Hsp90 inhibition in cells where MDM2 was knocked down by siRNA. For this, we monitored p53 levels in GA-treated HepG2 cells (wild-type p53) that were transfected with either control siRNA (si-control) or siRNA
Conformational Change Precedes p53 Proteasomal Degradation

against MDM2. As shown in Figs. 9, B and C, p53 levels decreased in si-control transfected HepG2 cells after GA treatment but did not decrease in cells where MDM2 was knocked down by siRNA. In total, results of Fig. 9 demonstrate Hsp90 inhibition promotes a decrease in wt p53 levels that is both MDM2- and proteasome-dependent.

DISCUSSION

Wild-type p53 is a short-lived and conformationally labile protein. Factors that control the conformation of p53 are likely to be important determinants of p53 activity. Hsp90 is a molecular chaperone protein, and there is abundant evidence that Hsp90 can maintain p53 in its wild-type conformation (10, 12). p53 displayed a wild-type or mostly wild-type conformation in cells when expressed alone. However, co-expression with MDM2 promoted an accumulation of p53 with a mutant conformation. This suggested MDM2 binding could drive wild-type p53 into a mutant conformation, at least to some extent. Strikingly, however, wild-type p53 was markedly shifted toward a mutant conformation when cells co-expressing p53 and MDM2 were treated with Hsp90 inhibitors (17-AAG, GA, radi- cicol). This effect required the MDM2 binding domain in the p53 N terminus and the p53-binding domain in the MDM2 N terminus but did not require the MDM2 RING-finger domain that is necessary for its ubiquitination activity. Over-expression of Hsp90 partially blocked the change in p53 conformation seen when cells expressing p53 and MDM2 were treated with GA. Together, these results suggest MDM2 and Hsp90 have opposing effects on p53 conformation. MDM2 binding to the p53 N terminus has the effect of driving wild-type p53 into a mutant conformation, whereas Hsp90 opposes this effect and maintains p53 in a wild-type conformation. How does MDM2-binding to the p53 N terminus alter p53 conformation? Recent studies have identified a secondary MDM2 binding site in the core domain of p53 that can bind the central acidic domain of MDM2 (32, 33). One possibility is that MDM2 binding to the p53 N terminus alters p53 conformation, at least to some extent.

Because MDM2 and Hsp90 have opposing effects on p53 conformation, we monitored p53 degradation by MDM2 in the presence or absence of Hsp90. We found that overexpression of Hsp90 could diminish p53 degradation by MDM2 in transfected cells, and conversely, that Hsp90 inhibitors (17-AAG, GA, radi-cicol). This effect required the MDM2 binding domain in the p53 N terminus and the p53-binding domain in the MDM2 N terminus but did not require the MDM2 RING-finger domain that is necessary for its ubiquitination activity. Over-expression of Hsp90 partially blocked the change in p53 conformation seen when cells expressing p53 and MDM2 were treated with GA. Together, these results suggest MDM2 and Hsp90 have opposing effects on p53 conformation. MDM2 binding to the p53 N terminus has the effect of driving wild-type p53 into a mutant conformation, whereas Hsp90 opposes this effect and maintains p53 in a wild-type conformation. How does MDM2-binding to the p53 N terminus alter p53 conformation? Recent studies have identified a secondary MDM2 binding site in the core domain of p53 that can bind the central acidic domain of MDM2 (32, 33). One possibility is that MDM2 binding to the p53 N terminus alters p53 conformation, at least to some extent.
Conformational Change Precedes p53 Proteasomal Degradation

Certain cancer-derived p53 mutant proteins display increased susceptibility to ubiquitination compared with the wild-type protein. Hupp and co-workers (35) reported that the increased susceptibility of mutant p53s to MDM2-mediated ubiquitination results from a conformational change in p53 (evidenced by exposure of the pAb-240 epitope) that causes exposure of lysine residues that can then be targeted for ubiquitination. Based on this, it is reasonable to suspect that the conformational change induced by MDM2 binding leads to the exposure of lysines in p53 that can then be ubiquitinated. In the current study wild-type p53 accumulated in a mutant (pAb-240 reactive) conformation when its proteasome-dependent degradation was blocked during recovery from stress treatment. In addition, wild-type p53 accumulated in a mutant conformation when its degradation by MDM2 was blocked by proteasome inhibitor treatment in transiently transfected cells. These findings indicate that wild-type p53 assumes a mutant conformation before its degradation by the proteasome. Ubiquitinated p53 also accumulated in a mutant conformation when p53 degradation by MDM2 was inhibited. Moreover, a p53-ubiquitin fusion protein believed to mimic ubiquitinated p53 had a mutant conformation in MDM2-null cells. This suggests p53 that is ubiquitinated in its C terminus maintains a mutant conformation even in the absence of MDM2 binding. Taken together, these results support a model in which MDM2 binding to the p53 N terminus induces a conformational change in p53. This conformational change most likely exposes lysine residues for ubiquitination and is opposed by Hsp90. The covalent attachment of ubiquitin may then serve to lock p53 in a mutant conformation in the absence of MDM2.

diverse stresses. In fact, a potential role for Hsp90 in p53 stabilization is suggested by previous studies in which Hsp90 inhibition by GA treatment diminished the stress-induced accumulation of p53 after heat shock or camptothecin treatment (11, 12). These findings suggested that Hsp90 may play a role in the stabilization of p53 in response to these and perhaps other stresses. However, any putative role for Hsp90 in p53 stability control is likely to be complex and dependent on multiple factors. Zylicz and co-workers (34) reported that purified Hsp90 had higher affinity for wild-type p53 than for the conformational mutant p53R175H (34). In contrast, complexes of Hsp90 and its co-chaperone partners (Hsc70, Hsp40, and Hop) bound preferentially to the mutant p53 and not to wild-type protein. Accordingly, binding between Hsp90 and p53 and subsequent effects on wild-type p53 stability is likely to be regulated by the levels of these co-chaperones and the association of Hsp90 with them.

REFERENCES


FIGURE 9. Hsp90 inhibition promotes proteasome and MDM2-dependent p53 degradation. A, MCF7 cells (wild-type p53) were mock-treated or treated with Hsp90 inhibitor (17-AAG or GA, 10 μM final concentration) for 1 or 2 h. Where indicated the cells were also treated with proteasome inhibitor MG132 (25 μM final concentration) or nutilin (30 μM final concentration). p53 levels are shown. Tubulin levels were used as the loading control. B, HepG2 cells (wild-type p53) were transfected with control siRNA or transfected with siRNA against MDM2 (siMDM2). 48 h after transfection, cells were treated with GA (10 μM) for 30 or 120 min. p53 and MDM2 levels were assessed by immunoblotting. Actin levels were used as a loading control. Similar results were obtained in two separate experiments. C, p53 levels from two separate experiments in B were quantified by densitometry using Image-J software. Average results are plotted.
Conformational Change Precedes p53 Proteasomal Degradation

MDM2 binding induces a conformational change in p53 that is opposed by heat-shock protein 90 and precedes p53 proteasomal degradation.

Mark Sasaki, Linghu Nie, and Carl G. Maki

In Fig. 4 we reported that the p53-ubiquitin fusion protein has a mutant conformation (pAb1620/pAb240). This p53-ubiquitin fusion protein has been used to mimic p53 mono-ubiquitinated in its C terminus. Subsequent to our manuscript being accepted, we discovered that the p53-ubiquitin fusion protein we used has a deletion of valine 218 near the pAb240 epitope. We have subsequently generated a new p53-ubiquitin fusion protein with valine 218 intact and compared its conformation to that of wild-type p53. This involved immunoprecipitation with the wild-type (pAb1620) and mutant (pAb240) conformation-specific antibodies followed by immunoblotting for p53. We did not detect an appreciable difference in conformation between wild-type p53 and the p53-ubiquitin fusion in these subsequent experiments. Therefore, the observation that the p53-ubiquitin fusion protein has an altered conformation compared with wild-type p53 was made in error. It remains possible that MDM2-mediated ubiquitination of p53, particularly at lysines within the p53 DNA-binding domain, could alter p53 conformation. However, the suggestion that C-terminal mono-ubiquitination might hold p53 in a mutant conformation is not supported.

Role of the cyclic AMP-dependent protein kinase in homologous resensitization of the β₁-adrenergic receptor.

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The images shown in Figs. 2A (p. 21138) and 4A (p. 21140) were obtained from slides prepared simultaneously from the same colony of cells. Panel c in Fig. 2A and panel K′ in Fig. 4A, due to an inadvertent error, were derived from slides that were not part of the set shown. The correct Fig. 2A, panel c, and Fig. 4A, panel K′, are shown below. The distribution of pixels in the revised panel c (Fig. 2A) and panel K′ (Fig. 4A) is similar to that in the corresponding published images. The legends for Figs. 2 and 4 and the text remain unchanged.

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