The Conformationally Flexible S9–S10 Linker Region in the Core Domain of p53 Contains a Novel MDM2 Binding Site Whose Mutation Increases Ubiquitination of p53 in Vivo*

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Although the N-terminal BOX-I domain of the tumor suppressor protein p53 contains the primary docking site for MDM2, previous studies demonstrated that RNA stabilizes the MDM2-p53 complex using a p53 mutant lacking the BOX-I motif. In vitro assays measuring the specific activity of MDM2 in the ligand-free and RNA-bound state identified a novel MDM2 interaction site in the core domain of p53. As defined using phage-peptide display, the RNA/MDM2 isoform exhibited a notable switch in peptide binding specificity, with enhanced affinity for novel peptide sequences in either p53 or small nuclear ribonucleoprotein-U (snRNP-U) and substantially reduced affinity for the primary p53 binding site in the BOX-I domain. The consensus binding site for the RNA/MDM2 complex within p53 is SGXXLGEESXF, which links the S9–S10 β-sheets flanking the BOX-IV and BOX-V motifs in the core domain and which is a site of reversible conformational flexibility in p53. Mutation of conserved amino acids in the linker at Ser261 and Leu264, which bridges the S9–S10 β-sheets, stimulated p53 activity from reporter templates and increased MDM2-dependent ubiquitination of p53. Furthermore, mutation of the conserved Phe270 within the S10 β-sheet resulted in a mutant p53, which binds more stably to RNA/MDM2 complexes in vitro and which is strikingly hyper-ubiquitinated in vivo. Introducing an Ala19 mutation into the p53F270A protein abolished both RNA/MDM2 complex binding and hyper-ubiquitination in vivo, thus indicating that p53F270A protein hyper-ubiquitination depends upon MDM2 binding to its primary site in the BOX-I domain. Together, these data identify a novel MDM2 binding interface within the S9–S10 β-sheet region of p53 that plays a regulatory role in modulating the rate of MDM2-dependent ubiquitination of p53 in cells.

The biochemical activity of p53 linked with tumor suppression is its function as a sequence-specific DNA binding protein and transcription factor that controls the expression of a large panel of gene products implicated in normal growth control, DNA repair, cell-cycle arrest, apoptosis, and angiogenesis (2). The stress-regulated transactivation function of p53 is driven by its sequence-specific DNA binding domain and is coordinated by specific protein-protein interactions that can in turn be modulated by covalent and non-covalent modifications. The central core domain of p53 from amino acids 90–295 contains the sequence-specific DNA binding domain (3). Inactivating mutations in p53 at over 200 different amino acid positions within this core DNA binding domain have been detected in human cancers (4), and the mutation often results in the unfolding and accumulation of p53 protein in the nucleus of the cancer cell (5). An oligomerization domain from amino acids 320–356 flanks the conserved core sequence-specific DNA binding domain and is required to assemble p53 into its tetrameric structure (6, 7).

Regulatory domains at the N and C termini of p53 modulate protein-protein interactions and DNA-protein interactions that affect the rate of p53-dependent transcription (8). The C terminus of p53 contains a negative regulatory domain whose phosphorylation at Ser215 by cyclin-dependent kinases (9) or acetylation at multiple lysine residues by p300 (10) stimulates the transcription activity of p53 in vivo. The N-terminal regulatory domain of p53 contains the highly conserved BOX-I domain that directs the binding of p53 to proteins, including the positive effector p300 and the inhibitor MDM2, the balance of which regulates the tumor suppressor activity of p53. Phosphorylation at Ser15, Thr18, and Ser20 can either reduce MDM2 binding (11, 12) or stabilize the p53-p300 transcription complex (13), resulting in a net activation of p53 function. Thus, the concerted activation of the sequence-specific DNA binding function of p53 by post-translational modification of its C-terminal domain and the kinase-dependent stabilization of the p53-p300 complex provide a working model to explain the basic mechanism of how p53-dependent gene expression can be activated.

By contrast to p53 activation reactions, inhibition of p53 activity is less understood but involves an MDM2-dependent pathway that functions in cycling cells to degrade p53 and keep its transcriptional activity relatively low. MDM2 protein contains at least four independent functional domains, including: 1) an N-terminal domain that recognizes the BOX-I domain of p53 (14); 2) a central domain that binds to the tumor suppressor protein p14ARF; 3) a putative zinc binding domain (15); and 4) a RING-finger RNA binding domain (16). The original localization of the primary MDM2 docking site on p53 protein in the
N-terminal BOX-I domain was defined using small peptide-mimetics (17), crystallographic analysis (18), and mutational studies (15, 19, 36). These studies revealed that a highly conserved hydrophobic series of amino acids within the N-terminal region of p53 from amino acids 14–27 interact with a hydrophobic binding pocket in the N-terminal domain of MDM2 protein. The microinjection of monoclonal antibodies to the p53 binding interface of MDM2 protein can activate p53-dependent expression providing direct evidence that MDM2 is normally a negative regulator of p53 function in vivo (20, 21). Scaffold proteins fused to the BOX-I domain of p53 can activate p53 function by virtue of binding to MDM2 protein (13, 22), providing additional evidence for the importance of this small BOX-I domain as an independent binding motif that is recognized by MDM2 protein in vivo.

Although MDM2 binding to the p53 tetramer results in polyubiquitination of the C-terminal domain of p53 and subsequent degradation, the BOX-I motif is itself sufficient for targeting a heterologous fusion protein for degradation by MDM2 protein (23). These latter data identify the minimal polypeptide fragment that can be modified by MDM2-dependent degradation machinery in vivo, but how MDM2 protein interacts with the native p53 tetramer is undefined. For example, although the BOX-I motif can promote degradation of a heterologous fusion protein in cells (13, 23), monomeric p53 protein, paradoxically, is not degraded by MDM2 (59) indicating that other determinants in the p53 tetramer regulate MDM2 binding in cells. A more recent study has shown that MDM2 protein, when complexed to RNA in vitro, can bind to p53 lacking the BOX-I domain, suggesting that RNA can induce a conformational change in MDM2 that directs its binding to a novel site in p53 (25). The purpose of this study was to use a biochemical approach to begin to reconstitute the MDM2-p53 tetramer complex in vitro and determine whether co-factors can modulate the in vitro stability of the MDM2-p53 complex. We provide direct evidence for a secondary docking site for MDM2 within the core domain of p53 that resides within a conformationally flexible S9–S10 β-sheet region flanked by conserved domains BOX-IV and BOX-V. Mutant forms of p53 that contain single point mutations at conserved amino acids within this S9–S10 β-sheet region have increased levels of MDM2-dependent ubiquitination in cells. This conformationally flexible motif is interestingly constrained in the wild-type p53 protein-DNA complex (3), but the S9–S10 β-sheet region is unfolded or denatured in mutant p53 protein within human cancers (5, 35). The denaturation or unfolding of p53 protein defined the first molecular defect of the p53 pathway in human cancer cells, and the refolding of p53 protein by small molecules has been the subject of recent screening programs aimed at reactivating mutant p53 protein function (60–62). Together, these data, identifying the conformationally flexible linker within the S9–S10 β-sheet region of p53 protein as a novel MDM2 binding interface, suggest an interplay may exist between p53 protein unfolding and factors that modulate the rate of MDM2-dependent ubiquitination of p53 in vivo.

**EXPERIMENTAL PROCEDURES**

**Purification of Full-length Human MDM2 Protein**—All reagents were from Sigma Chemical Co. unless indicated otherwise. Bacterially expressed p53 was purified as described previously (24). Full-length human MDM2 was expressed in Escherichia coli BL21 cells (25, 27) and MDM2 was induced by addition of 1 mM final concentration of isopropl-1-thio-β-D-galactopyranoside. Harvested cells were washed with ice-cold 50 mM Tris-HCl (pH 8.0), and the final pellet was resuspended at 0.66 g/ml in 10% sucrose, 50 mM Tris-HCl (pH 8.0), followed by the addition of lysozyme (150 μM/ml final concentration) and NaCl (0.15 M final concentration). The cell suspension was incubated in an ice bath for 45 min, then warmed to 37 °C for 1 min and returned to 0 °C. The cells were lysed by ice sonication, after the addition of Pefabloc (2 mM, Roche Molecular Biochemicals), DTT (5 mM, BDH Laboratory Supplies), and benzamidine (1 mM). The lysate was centrifuged, and the supernatant was fractionated on a 5-ml HiTrap-SP column (Amersham Biosciences, Inc.) equilibrated with buffer A (1% glycerol, 25 mM HEPES, pH 8.0, 0.02% Triton X-100, 150 mM NaCl, and 1 mM benzamidine). The supernatant was diluted with buffer B prior to application to the column, and bound protein was eluted in a linear gradient in buffer A from 0.05 to 1.0 M KCl. Aliquots (1 μl) of the fractions from the column were assayed for the purity of MDM2 by SDS gels and presence of MDM2 by Western blotting. To determine the oligomerization state of purified MDM2 protein was applied onto size-exclusion high-performance liquid chromatography (Superose 12 HR 10/30, Amersham Biosciences, Inc.) with buffer B (10 mM KCl, 25 mM HEPES, pH 7.4). The QuikChange site-directed mutagenesis kit (Stratagene) was used to create p53 mutants, with primers designed to change Phe39, Ser208, Leu214, and Phe270 into alanine.

**Immunological Assays**—The peptide or p53 tetramer binding activity of MDM2 was examined by ELISA, as described previously (25, 26). Essentially, 96-well plates (Dynex Microlite 2) were first coated with p53, MDM2, or streptavidin and the indicated biotinylated peptide for 16 h, as described previously (13, 26). Non-reactive sites were blocked in 3% bovine serum albumin in PBS-Tween 20 (0.02% v/v) to reduce the nonspecific binding. This was followed by titrating increasing concentrations of p53, MDM2, peptides, or RNA-binding serum albumin in PBS-Tween 20 (0.02% v/v) for 1 h, followed by an extensive wash, and incubation with the indicated IgG. All reactions were carried out at 4 °C and detected by the appropriate secondary antibody linked to horseradish peroxidase from DAKO. The signal detection by enhanced chemiluminescence was developed using Fluoropass Ascent FL. The human p14ARF domain peptide contained the sequence MVRRPLVLHRRLBQGGPPVY (27). The BOX-I domain of p53 contains amino acids 14–27, as described previously (13). The DO-12 epitope peptide used contained the sequence SGNLGLRNSFEVRV-CACPGGRDR. Phage-peptide display, on antibody-captured bacterially expressed MDM2 protein or bacterially expressed MDM2 protein reconstituted with RNA (poly(rG)), was carried out using three rounds of phage-selected selection as described previously for purified phage-specific monoclonal antibodies toward phospho-epitopes of p53 (9, 28).

**p53 and MDM2 Co-immunoprecipitation**—Immunoprecipitation was performed using 5 μl of wild-type or mutant p53 proteins translated in TnT Quick Coupled Transcription/Translation systems (Promega) with 10 μl of MDM2, which was purified by the method indicated above (SP-fraction). Rabbit reticulocyte lysates containing translated p53 protein were incubated at 1 h at room temperature in 50 μl of Immunoprecipitation buffer (1% Triton X-100, 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and protease inhibitors) with Protein G-Sepharose 4 Fast Flow (Amersham Biosciences, Inc.), the p53 monoclonal antibody PAb421, in the absence or presence of MDM2 protein (SP-fraction) and in the absence or presence of 1 μg of RNAse (Sigma). The Protein G was washed four times with immunoprecipitation buffer, and bound proteins were eluted in 20 μl of SDS sample buffer. The eluted samples (10 μl) were loaded onto SDS-acrylamide gel and blotted for MDM2 using the monoclonal antibody 2A10.

**Transfection Assays**—H1299 p53−/− cells and SAOS-2 p53−/− cells were incubated in RPMI 1640, and Dulbecco’s modified Eagle’s medium, respectively, supplemented with 10% fetal calf serum. Cells were cultured at 37 °C, 5% CO2 in a humidified atmosphere. 1 × 10⁶ of H1299 cells, A375 cells, or 4.5 × 10⁶ of SAOS-2 cells were seeded on 6-cm tissue culture plates and transfected using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer’s protocol, and p53-dependent transcription was carried out as described previously (13). Briefly, SAOS-2, H1299, or A375 cells were harvested 24 h post-transfection and lysed in reporter lysis buffer. The corresponding luciferase and β-galactosidase assays were carried out according to the manufacturer’s protocol (Promega). The exact quantity of DNA transfected is indicated in each experiment, and, where necessary, carrier DNA was transfected to keep the same amount of DNA constituent in each transfection. For detection of protein by Western blot analysis, 24 h post transfection, the cells were harvested and lysed in Nonidet P-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1% Nonidet P-40, 2 mM dithiothreitol (DTT) containing a protease inhibitor mixture). Protein
concentration was determined by Bradford assay, and an equal amount of total protein was loaded onto the SDS gels. p53-ubiquitination assays using p53 transfected into H1299 cells were carried out as described previously (29). For detection of ubiquitinated p53, 24 h-post transfection, H1299 cells were treated with the proteasome inhibitor N-acetyl-Leu-Leu-norleucinal at a final concentration of 50 μM for 4 h prior to lysis using lysis buffer HS (100 mM Tris-HCl (pH 8.0) and 100 mM DTT, containing a protease inhibitor mixture). When the half-life of p53 protein (and indicated mutants) was examined, H1299 cells were transfected with 1 μg of p53 DNA (wild-type or mutant p53) in the absence or presence of 1 or 3 μg of MDM2 expression DNA. Cells were treated with 1.33 mM of the protein-synthesis inhibitor cycloheximide (SPELCO) 24 h post-transfection and harvested at the indicated time points.

RESULTS

Purification of Full-length MDM2 Protein—Human MDM2 protein contains an N-terminal domain that binds to the N-terminal BOX-I domain of p53 and C-terminal sub-domains that interact with zinc, RNA, and the tumor suppressor protein p14ARF (30). Although MDM2 protein is known to bind to the N-terminal BOX-I domain of p53, a recent study has shown that RNA binding by MDM2 can promote MDM2 protein interaction with a BOX-I deletion mutant of p53 protein using surface plasmon resonance (25). These latter data suggest that RNA induces a conformational change in MDM2 protein via the RING finger domain and that this promotes an interaction with p53 outside the canonical BOX-I motif. This current study was set up initially to determine in fact whether ligand binding to the C-terminal domain of MDM2 could change its conformation and whether this change directs MDM2 to interact with a previously unidentified motif within the core domain of p53. To begin to address this possibility, full-length human MDM2 protein was first purified in a native state from bacterial expression systems. Following lysis using gentle perturbations that can maintain recombinant protein conformational integrity, a purification of MDM2 protein was developed using standard chromatographic methods. The best purification was obtained using cation exchange chromatography, where MDM2 protein eluted as a triple of three protein bands at a conductivity equivalent to 0.4–0.5 X KCl (Fig. 1A, lanes 1–4). Immunoblotting of the peak fractions using distinct monoclonal antibodies confirms that the triplet protein bands of interest are MDM2 protein (Fig. 1B, lanes 1–4). It is not known whether the three protein bands stem from alternative translational reading and termination of the mdm2 transcript in E. coli or from proteolysis of MDM2 protein. In addition, because MDM2 protein has a predicted molecular mass of ~55 kDa yet migrates in a denaturing gel at a molecular mass of greater than 90 kDa, altered protein-folding pathways utilized during its translation may produce isoforms that migrate anomalously in a denaturing SDS-gel.

The MDM2 protein eluted as a monomer on gel filtration (data not shown) indicating that native MDM2 protein purified from bacteria is not a large oligomeric inclusion body intermediate. The specific activity of the native fraction of full-length MDM2 protein was tested in a BOX-I peptide binding assay and in a p53-tetrameric binding assay to determine whether MDM2 was active when purified under these conditions. When MDM2 was titrated into streptavidin-ELISA wells pre-coated with a biotinylated BOX-I domain peptide, a dose-dependent increase in MDM2 binding was observed using two different monoclonal antibodies as a detection system (Fig. 1C). MDM2 protein was also active in binding to native p53 tetramers (Fig. 1D), indicating that this fraction of monomeric MDM2 is fully competent in peptide or protein binding assays. However, differences in the specific activity of MDM2 protein toward p53 tetramers, but not the BOX-I peptide, depend on the monoclonal antibody used as the detection system (Fig. 1, D versus C). These latter data suggest that either mAb 4B2 de-stabilizes
the p53 tetramer-MDM2 protein complex or that mAb 2A10 stabilizes the p53 tetramer-MDM2 protein complex.

RNA Can Stabilize the MDM2-Tetrameric p53 Protein Complex—The functional domains of MDM2 protein include: (i) the N-terminal p53 binding site (amino acids 19–102); (ii) the N-terminal p14ARF binding site (amino acids 212–244); (iii) the nuclear localization signal (amino acids 181–185); (iv) the nuclear export sequence (amino acids 197–205); (v) a C-terminal zinc finger; (vi) a C-terminal RING finger domain; and (vii) a nucleolar localization signal sequence (amino acids 466–473). A quantitative ELISA was used to determine whether ligands that bind to the C-terminal domain of MDM2 change the stability of the tetrameric p53-MDM2 protein complex. Ligands included RNA (polyrG), which binds to the extreme C-terminal RING finger domain, zinc that binds to the more N-terminal zinc binding site, and p14ARF, which binds to the central domain of MDM2.

The first sequence of events involved analyzing the affects of RNA on the specific activity of MDM2 protein, which included adsorption of MDM2 protein onto the solid phase, followed by incubation of MDM2 protein with RNA, and ending with the incubation of the ligand-free form of MDM2 or the MDM2-RNA complex with native p53. The RNA used was the homopolyribonucleotide polyrG, which binds with a high affinity to MDM2 protein (16). Under these conditions, a dose-dependent increase in the amount of p53 bound to MDM2 occurs after the addition of 100 pg to 10 ng of RNA (Fig. 2A). When the sequence of events was changed by first capturing MDM2 protein with the 4B2 monoclonal antibody, a similar trend was observed in that the addition of RNA to MDM2 enhanced the stability of the p53-MDM2 protein complex (Fig. 2B). However, an increase in the amount of p53 bound to MDM2 increases after the addition of 100 ng to 10 μg of RNA, resulting in a 1000-fold reduction in the sensitivity of MDM2 to RNA. Because the mAb 4B2 was also less intrinsically efficient in capturing the p53-MDM2 protein complex than mAb 2A10 (Fig. 1), the 4B2 capture method of quantitating the MDM2-p53 complex was not used further.

Because the conformation of p53 is known to change upon binding to nucleic acid (31, 32), a control was performed to determine whether the effects of RNA were due to changes in MDM2 conformation rather than that of p53. The sequence of events included adsorption of p53 tetramers onto the solid phase, followed by the incubation of p53 protein with RNA, and ending with the incubation of the p53-RNA complex with native MDM2 protein. Under these conditions, a decrease in the amount of p53 bound to p53 occurs after the addition of 100 pg to 10 ng of RNA (Fig. 2C). These data contrast with the affects of RNA on stabilizing the MDM2-p53 complex (Fig. 2, A and B) and are consistent with the observation that p53-DNA complexes exhibit reduced binding to MDM2 (25).

RNA Can Destabilize the Formation of the MDM2-p53 BOX-I Domain Complex—Experiments were next carried out to determine whether stabilization of the MDM2-p53 complex by RNA involves changes in the interaction of MDM2 protein with the BOX-I domain of p53 and/or an interaction of MDM2 with a novel site in the core domain of p53. For example, RNA may stabilize the MDM2-p53 complex by increasing the affinity of MDM2 protein for the N-terminal BOX-I domain of p53. The specific activity of MDM2 protein was thus subsequently tested in a BOX-I peptide binding assay, because the use of a small p53-derived peptide also minimizes the affects other regions of p53 that bind either RNA, zinc, or p14ARF.

Strikingly, a preincubation of MDM2 protein with RNA reduced substantially the formation of the MDM2-BOX-I peptide complex (Fig. 3A). The addition of magnesium/ATP had no affect on the stability of the MDM2-BOX-I domain complex, whereas the addition of either zinc or p14ARF also reduced the stability of the MDM2-BOX-I domain complex (Fig. 3A) but not to the same extent. These data indicate that three distinct C-terminal binding ligands of MDM2 can reduce stability of the MDM2-BOX-I peptide complex and suggest that the ability of RNA-MDM2 complexes to bind to p53 protein coincides with a conformational change in the ligand-bound form of MDM2 pro-
tein (see below). These data are also consistent with the observation that MDM2/H18528 RNA complexes can bind stably to p53 tetramers lacking the N-terminal BOX-I domain (25). The stability of the MDM2/H18528 p14ARF complex was not similarly reduced or enhanced by the preincubation of MDM2 with either ATP, RNA, or zinc (Fig. 3B).

Together, these data indicate that the binding of MDM2 protein to its ligands can affect its specific activity as a p53 binding protein. The fact that RNA can simultaneously stabilize the p53/H18528 MDM2 protein complex and reduce the stability of the MDM2/H18528 BOX-I domain complex is internally consistent. However, the reduced stability of the MDM2/H18528 BOX-I domain complex in the presence of zinc (Fig. 3) is not compensated by an increase in the stability of the MDM2-tetrameric p53 complex in the presence of zinc (data not shown). These latter data suggest that RNA or zinc induce distinct conformational changes in MDM2 protein. Partial proteolysis was used to determine whether in fact evidence could be found for a difference in the conformation of the RNA-MDM2 protein complex and the zinc-MDM2 protein complex. The first analysis was designed to examine the proteolytic cleavage products of MDM2 protein in the absence or presence of RNA using Trypsin (Fig. 4A). A titration of Trypsin into reactions containing MDM2 protein in the presence of RNA (Fig. 4A, lanes 6–8) gave rise to a different pattern of proteolytic products than in reactions without RNA (Fig. 4A, lanes 2–4). Most notable was the resistance of the 27-kDa fragment to proteolysis in the presence of RNA (Fig. 4A, lanes 7 and 8 versus lanes 3 and 4). Using the protease Glu-C, a similar resistance to proteolysis of a 53-kDa fragment was observed when MDM2 protein was incubated with RNA (Fig. 4B, lanes 4 and 5 versus lanes 2 and 3). Finally, although RNA and zinc reduce the binding of MDM2 protein to the BOX-I domain, a resistance to proteolysis of a 52- and a 45-kDa fragment was uniquely observed when MDM2 protein was incubated with zinc (Fig. 4B, lanes 6 and 7 versus lanes 1–5). Together, these data suggest that the ability of RNA to stabilize the MDM2-tetrameric p53 complex and to inhibit the MDM2-BOX-I peptide complex (Fig. 3A) may be due to a conformational change in the MDM2 protein that is distinct from that induced by zinc.

**Fig. 3. Ligand binding by MDM2 destabilizes the MDM2-BOX-I domain complex.** A, MDM2/p53 BOX-I domain stability. BOX-I domain peptides derived from p53 were fixed in ELISA wells as described previously (13, 26). Briefly, 1 μg of biotinylated BOX-I domain peptides were immobilized to streptavidin coated 96-well plates. MDM2 (7 ng) was incubated with ligands (1 μg of polyrG, 1 mM ATP·S (Calbiochem), 1 mM zinc sulfate, and 0.8 μg of p14ARF) for 1 h, and then these mixtures were added to the BOX-I domain-coated wells. The excess MDM2 was washed away using PBS-Tween 20 (0.02% v/v). The monoclonal antibody SMP14 was subsequently added into the reactions, and the amount of MDM2 protein bound to the BOX-I domain was then quantitated using a peroxidase-coupled anti-mouse IgG and enhanced chemiluminescence. The data are depicted as luminescence in RLU (a percentage of the control) as a function of the MDM2 isoform. B, MDM2/p14ARF domain stability. p14ARF domain peptides derived from the N-terminal domain of p14ARF were fixed in ELISA wells. Briefly, 1 μg of biotinylated p14ARF domain peptides was immobilized to streptavidin-coated 96-well plates. MDM2 (7 ng) was incubated with ligands (1 μg of polyrG, 1 mM ATP·S (Calbiochem), and 1 mM of zinc sulfate) for 1 h, and then these mixtures were added to the p14ARF domain-coated wells. The excess MDM2 was washed away using PBS-Tween 20 (0.02% v/v). The monoclonal antibody SMP14 was subsequently added into the reactions, and the amount of MDM2 protein bound to the p14ARF domain was then quantitated using a peroxidase-coupled anti-mouse IgG and enhanced chemiluminescence. The data are depicted as luminescence in RLU (a percentage of the control) as a function of the MDM2 isoform.
The difficulty in predicting the putative secondary docking site for the RNA-MDM2 isoform in the core domain of p53 resulted in two distinct approaches. The first involved using phage-peptide display to identify novel peptide domains that bind to the RNA-MDM2 isoform and determining using a search engine whether such domains have a homology site in common with the core domain of p53. A second approach was to determine using a search engine whether the tumor suppressor protein pRb, which contains an atypical and undefined interaction site for MDM2 (33), has a motif in common with the core domain of p53.

Localization of the Secondary Binding Site on p53 for MDM2 Protein—The difficulty in predicting the putative secondary docking site for the RNA-MDM2 isoform in the core domain of p53 resulted in two distinct approaches. The first involved using phage-peptide display to identify novel peptide domains that bind to the RNA-MDM2 isoform and determining using a search engine whether such domains have a homology site in common with the core domain of p53. A second approach was to determine using a search engine whether the tumor suppressor protein pRb, which contains an atypical and undefined interaction site for MDM2 (33), has a motif in common with the core domain of p53.

In the first instance, the ligand-free form of MDM2 or the MDM2-RNA isoform was adsorbed on to the solid phase by capture using a MDM2-specific monoclonal antibody and subjected to three rounds of phage-peptide display using a 12-mer peptide library, as described previously for two phospho-specific monoclonal antibodies toward p53 (9, 28). Phage-peptide display is a versatile technique that can give rise to high affinity peptide ligands that contain homology to bona fide binding proteins. For example, phage-peptide display analysis using the ligand-free form of full-length MDM2 protein yielded a series of peptides with high homology to the BOX-I domain of p53 (Fig. 5A). The high affinity binding phage were purified and sequenced giving rise to two distinct classes of peptides. The first class was obtained using ligand-free full-length MDM2 protein (Fig. 5A) and had homology to the BOX-I domain of p53 at residues critical for MDM2 binding (Phe\(^{19}\)X-X-Trp\(^{20}\)-X-X-Leu\(^{26}\)) (18). These results are identical to the phage-peptide display performed on the N-terminal fragment of MDM2 protein fused to GST (34), except our analysis also included the Thr\(^{18}\) residue as being an important component of MDM2 binding giving rise to the consensus sequence Thr\(^{18}\)-Phe\(^{19}\)-X-X-Trp\(^{20}\)-X-X-Leu\(^{26}\) (Fig. 5A). These latter data are consistent with the pronounced instability of the MDM2/p53 BOX-I domain complex containing a phosphate residue at Thr\(^{18}\) (26). A distinct class of peptides were obtained using the MDM2-RNA isoform (Fig. 5A). A protein sequence with high homology to snRNP-U was obtained and no peptides were obtained with homology to the BOX-I domain of p53. These data are consistent with the observation that RNA changes the conformation of MDM2 (Fig. 4) and that MDM2-RNA complexes can bind to p53 lacking the BOX-I domain (25). When snRNP-U and p53 were scanned for similar sites of homology, a motif with a significant degree of homology was found in between the conserved BOX-IV and BOX-V domains of p53 (Fig. 5B, SXGLLGESXF). As an independent approach, the C-terminal domain of pRb was scanned for putative homology motifs within the central coding region of p53, because this region of pRb has a non-canonical and undefined MDM2 docking site (33). A striking degree of primary amino acid homology was observed between p53, snRNP-U, and pRb, with the p53 motif again residing in between the conserved BOX-IV and BOX-V domains (Fig. 5B).

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A final prediction of these studies would be that ligand-free MDM2 protein would bind better to the BOX-I domain and display little affinity for the DNA complex (89–91 linker peptide) containing the sequence SQNLGGRNSF. By contrast, the RNA-MDM2 complex would be predicted to switch
served to p53 protein. A motif in the central domain of p53 in between con-
screened using the E-MOTIF search engine for domains with homology
region of pRb and the entire coding region of snRNP-U were both were
sor protein pRb binds to MDM2 protein at an undefined site (33). This
sive MDM2 binding site. The C-terminal domain of the tumor suppres-
form gave rise to the consensus sequence that matched most closely
sequence to determine the insert peptide sequence. The ligand-free
form of MDM2 protein gave rise to the general consensus derived from
BOX-I domain of p53 (as indicated), whereas the RNA-DM2 iso-
isoform gave rise to the consensus sequence that matched most closely
SNLGRNSF motif can be di-
sended ubiquitination. The SGNLLGRNSF motif can be di-
vided into two regions: 1) the SGNLLGRNSF motif is contained in the

A. Phage-peptide displays identifies a novel ligand-depen-

dent consensus site for MDM2

a. Ligand-Free MDM2

Φ consensus

p53

X TE F E L X X

Q E T E L D W L K L L P E

b. RNA-DM2

Φ consensus

snRNP-U

A S S L L X M X S F X

A S S L L T M E S F

B. Consensus site for the MDM2-RNA Complex

P53

SG N L L G R N S F

Rb

SK H L P G E S K F

Weel

SV K L R G S S L F

snRNP-U

SG M L L G E S S F

CON.

SG XL L L G E S S F

FIG. 5. Phage-peptide display identifies a novel RNA-responsive MDM2 binding motif. A, phage-peptide consensus sequences that bind to ligand-free MDM2 and RNA-bound MDM2. MDM2 protein or MDM2-RNA complexes were coated onto ELISA wells (as indicated in Fig. 2) and subjected to phage-peptide display using a 12-mer peptide library as described previously (9, 28). Briefly, native MDM2 protein (SP-Sepharose fraction, Fig. 1) or the RNA-DM2 isoform were captured onto ELISA wells with an anti-MDM2 monoclonal antibody as described in Fig. 2 legend. Following an incubation with the naïve phage-peptide library, elution of the phage with acid, and propagation of eluted phage, three rounds of amplification were carried out to acquire phage that bind with a high affinity to the indicated isoform of MDM2. The peptide inserts in each phage were tested for specificity and sequenced to determine the insert peptide sequence. The ligand-free form of MDM2 protein gave rise to the general consensus derived from the BOX-I domain of p53 (as indicated), whereas the RNA-DM2 isoform gave rise to the consensus sequence that matched most closely snRNP-U (as indicated). B, putative consensus site for the RNA-responsive MDM2 binding site. The C-terminal domain of the tumor suppressor protein pRb binds to MDM2 protein at an undefined site (33). This region of pRb and the entire coding region of snRNP-U were both screened using the E-MOTIF search engine for domains with homology to p53 protein. A motif in the central domain of p53 in between conserved BOX-IV and BOX-V domain from amino acids 261 to 270 was found to have significant homology to pRb, snRNP-U, and Weel1, to give rise to the general consensus site SGXLGGSXF.

specificity from the BOX-I domain and bind with a higher affinity to the S9–S10 linker peptide containing the SGNLGRNSF polypeptide sequence. Such a change in specificity is observed experimentally, with the MDM2-BOX-I domain peptide (left three panels) derived from p53 were titrated and fixed in ELISA wells as described under “Experimental Procedures.” Increasing amounts of peptide were preincubated with MDM2 bound to the indicated ligand (no addition, RNA, or zinc) and added into the ELISA wells in a stabilizing buffer. The monoclonal antibody 2A10 was subsequently added into the reactions and the amount of MDM2 protein bound to the BOX-I domain or the S9–S10 Linker peptide

loop that bridges the S9 and S10 β-sheets (Fig. 6C) and 2) the GESXF motif is contained within the S10 β-sheet (Fig. 6C). Marked deletion or multiple mutation of this linker region or the S10 β-sheet was not performed, because this may unfold

FIG. 6. Identification of an RNA-DM2 binding site in the central domain of p53. A, p53-DNA complexes destabilize the epitopes for the conformationally sensitive monoclonal antibodies. The control monoclonal antibodies (DO-1 and PAB241) and the conformationally sensitive monoclonal antibodies that bind to distinct epitopes in the core domain (DO-12 and PAB240) were absorbed onto ELISA wells, and the indicated p53 forms were added to the ELISA wells: p53 (white); p53-DNA complexes (black); p53R175H protein (dark gray); and p53R175H-DNA complexes (light gray). The amount of p53 captured by the indicated antibodies was then quantitated using a peroxidase-coupled anti-p53 polyclonal IgG (CM1) and tetramethyl-
benzidine (Kirkegaard & Perry Laboratories). The data are depicted as absorbance as a function of the monoclonal antibody used to capture p53. B, ligand binding by MDM2 changes its binding affinity for distinct domains of p53. BOX-I domain peptides (right three pan-
els) and the S9–S10 Linker peptide contained within the DO-12 epitope peptide (left three panels) derived from p53 were titrated and fixed in ELISA wells as described under “Experimental Procedures.” Increasing amounts of peptide were preincubated with MDM2 bound to the indicated ligand (no addition, RNA, or zinc) and added into the ELISA wells in a stabilizing buffer. The monoclonal antibody 2A10 was subsequently added into the reactions and the amount of MDM2 protein bound to the BOX-I domain or the S9–S10 Linker peptide domain was then quantitated using a peroxidase-coupled anti-mouse IgG and enhanced chemiluminescence. The data are depicted as luminescence in RLU as a function of the MDM2 isoform. C, the consensus RNA-MDM2 binding site is located in a conformationally constrained loop (amino acids 261–264, as indicated) that joins the S10 β-sheet region (as indicated by black). This loop + β-sheet region also contains the epitope for the monoclonal antibody DO-12, which represents a conformationally flexible region in the central domain of p53 (3, 35).
The S9–S10 linker mutants encoded by the p53S261A and p53L264A alleles are active as sequence-specific transcription factors. Expression vectors encoding wild-type p53, p53S261A, and the p53L264A (1, 2, or 5 μg of DNA, as indicated) were co-transfected into Saos-2 cells with the (A) p21-luciferase and β-galactosidase reporter DNAs or (B) the bax-luciferase and β-galactosidase reporter DNAs. The activity of the p53 is expressed as the ratio of chemiluminescence from the reporter vector to the internal β-galactosidase control vector.

The S9–S10 linker mutants encoded by the p53S261A and p53L264A alleles exhibit increases in MDM2-dependent ubiquitination. Expression vectors encoding wild-type p53, p53S261A, or p53L264A alleles (as indicated; 1 μg of DNA) and either without MDM2 (from left; lanes 1–3) or with the MDM2 encoding vector (from left; lanes 4–6) were transfected into H1299 cells, and the cell lysates were immunoblotted to examine for changes in their amount of ubiquitination products by immunoblotting with antibodies to p53 (DO-1), as described previously (29). The ladder of protein bands above the full-length p53 position is more pronounced with the two linker mutants.

In summary, the characterization of these three mutant proteins in vivo with alanine substitutions in the central MDM2

![Figure 7](image7.png)

**Figure 7**. The S9–S10 linker mutants encoded by the p53S261A and p53L264A alleles are active as sequence-specific transcription factors. Expression vectors encoding wild-type p53, p53S261A, and the p53L264A (1, 2, or 5 μg of DNA, as indicated) were co-transfected into Saos-2 cells with the (A) p21-luciferase and β-galactosidase reporter DNAs or (B) the bax-luciferase and β-galactosidase reporter DNAs. The activity of the p53 is expressed as the ratio of chemiluminescence from the reporter vector to the internal β-galactosidase control vector.

![Figure 8](image8.png)

**Figure 8**. The S9–S10 linker mutants encoded by the p53S261A and p53L264A alleles exhibit increases in MDM2-dependent ubiquitination. Expression vectors encoding wild-type p53, p53S261A, or p53L264A alleles (as indicated; 1 μg of DNA) and either without MDM2 (from left; lanes 1–3) or with the MDM2 encoding vector (from left; lanes 4–6) were transfected into H1299 cells, and the cell lysates were immunoblotted to examine for changes in their amount of ubiquitination products by immunoblotting with antibodies to p53 (DO-1), as described previously (29). The ladder of protein bands above the full-length p53 position is more pronounced with the two linker mutants.
interaction site has revealed differing degrees of enhancement of ubiquitination. Although the alanine substitution is a common change made in a protein to bring about a reduction in the stability of a protein/protein interaction, there have been instances where stabilization of a protein for its docking site by alanine mutation occurs (58, 63). These data suggest that some protein-protein interactions have evolved to maintain a lower affinity, but allowing reversibility in binding, and that binding affinity can be improved artificially. Such an improvement has been reported with the MDM2-p53 BOX-I complex, where mutation has led to the improvement on MDM2-peptide binding affinity by many orders of magnitude (22, 34).

We subsequently investigated whether the hyper-ubiquitination of p53F270A mutant protein was MDM2-dependent in vivo by examining the levels of ubiquitination using the p53F19A/F270A double mutant (Fig. 11). The hyper-ubiquitination of the p53F270A protein (Fig. 11, lane 1) was prevented by the additional Ala19 substitution (Fig. 11, lane 2). Furthermore, the p53F19A/F270A protein was not ubiquitinated after co-transfection of MDM2 (Fig. 11, lanes 3 and 5), under conditions where the p53F270A mutant protein showed increases in the ubiquitination pattern (Fig. 11, lanes 4 and 6). MDM2 protein levels were similar after transfection of the p53F270A mutant protein or the p53F19A/F270A double mutant protein (Fig. 11B), indicating that changes in MDM2 protein levels cannot explain the increases in p53F270A mutant protein ubiquitination. It is interesting, therefore, that wild-type p53 requires transfected MDM2 to catalyze its ubiquitination, whereas the p53F270A protein can rely on very small levels of endogenous MDM2 for its ubiquitination.

We next examined whether the hyper-ubiquitination of p53F270A mutant protein was due to increases in MDM2 binding affinity for the mutant and whether the half-life of the hyper-ubiquitinated p53F270A protein was MDM2-dependent. First, various p53 mutants were synthesized in reticulocyte lysates (Fig. 12) to determine whether MDM2 protein (SP fraction, Fig. 1) binds with a higher affinity due to this alanine substitution either the p53F270A allele (lanes 1, 2, and 3) or the p53F19A/F270A double mutant (Fig. 11). The hyper-ubiquitination of the mutant encoded by the inactive p53F270A allele is MDM2-dependent. Expression vectors encoding either the p53F270A allele (lanes 1, 2, and 3) or the p53F19A/F270A double mutant allele (lanes 4, 5, and 6) were transfected into H1299 cells, and the expressed proteins were examined for changes in their ubiquitination products by immunoblotting with antibodies to p53 (top panel). The ladder of protein bands below and above the full-length p53 position is more striking with the p53F270A allele and to a lesser extent with the p53L264A allele. The amount of endogenous MDM2 protein induced (middle panel) or endogenous p21 protein produced (bottom panel) with the indicated p53 transfections is shown using the indicated antibodies to MDM2 and p21, respectively.

We subsequently investigated whether the hyper-ubiquitination of the p53F270A allele is hyper-ubiquitinated in the absence of transfected MDM2. Expression vectors encoding wild-type p53, p53F270A, p53L264A, and p53F270A alleles (0.5, 1, and 2 μg of DNA, as indicated) were transfected into H1299 cells, and the expressed proteins were examined for changes in their steady-state levels of p53 and amount of ubiquitination products by immunoblotting with antibodies to p53 (top panel). The ladder of protein bands below and above the full-length p53 position is more striking with the p53F270A allele and to a lesser extent with the p53L264A allele. The amount of endogenous MDM2 protein induced (middle panel) or endogenous p21 protein produced (bottom panel) with the indicated p53 transfections is shown using the indicated antibodies to MDM2 and p21, respectively.

We subsequently investigated whether the hyper-ubiquitination of the mutant encoded by the inactive p53F270A allele is MDM2-dependent. Expression vectors encoding either the p53F270A allele (lanes 1, 2, and 3) or the p53F19A/F270A double mutant allele (lanes 4, 5, and 6) were transfected into H1299 cells, and the expressed proteins were examined for changes in their ubiquitination products by immunoblotting with antibodies to p53 (A). The ladder of protein bands below and above the full-length p53 position is more striking with the p53F270A allele. B, the amount of endogenous MDM2 protein induced (lanes 1 and 2) or transfected MDM2 levels (lanes 3–6) with the indicated p53 transfections is shown using the antibody to MDM2.

FIG. 9. The S10 β-sheet mutant encoded by the p53F270A allele is hyper-ubiquitinated in the absence of transfected MDM2. Expression vectors encoding wild-type p53, p53F270A, p53L264A, and p53F270A alleles (0.5, 1, and 2 μg of DNA, as indicated) were transfected into H1299 cells, and the expressed proteins were examined for changes in their steady-state levels of p53 and amount of ubiquitination products by immunoblotting with antibodies to p53 (top panel). The ladder of protein bands below and above the full-length p53 position is more striking with the p53F270A allele and to a lesser extent with the p53L264A allele. The amount of endogenous MDM2 protein induced (middle panel) or endogenous p21 protein produced (bottom panel) with the indicated p53 transfections is shown using the indicated antibodies to MDM2 and p21, respectively.

FIG. 10. The S10 β-sheet mutant encoded by the p53F270A allele is inactive in a p53-dependent transactivation assay. Expression vectors encoding wild-type p53, p53F270A, p53L264A, p53F270A, p53F270A, p53F270A, p53F270A (1, 2, or 5 μg of DNA, as indicated) were co-transfected into A375 cells with the p21-luciferase and β-galactosidase reporter DNAs. The activity of the p53 is expressed as the ratio of chemiluminescence from the reporter vector to the internal β-galactosidase control vector.

FIG. 11. Hyper-ubiquitination of the mutant encoded by the inactive p53F270A allele is MDM2-dependent. Expression vectors encoding either the p53F270A allele (lanes 1, 2, and 3) or the p53F270A double mutant allele (lanes 4, 5, and 6) were transfected into H1299 cells, and the expressed proteins were examined for changes in their ubiquitination products by immunoblotting with antibodies to p53 (A). The ladder of protein bands below and above the full-length p53 position is more striking with the p53F270A allele. B, the amount of endogenous MDM2 protein induced (lanes 1 and 2) or transfected MDM2 levels (lanes 3–6) with the indicated p53 transfections is shown using the antibody to MDM2.
The mutant encoded by the p53F270A allele binds to MDM2 better than wild-type p53 protein in an RNA-dependent manner. The indicated p53 alleles (lane 1, vector only; lane 2, wt p53; lane 3, p53F270A; lane 4, p53L264A; lane 5, p53F270A; lane 6, p53F19A/F270A; lane 7, p53R175H) were added to reticulocyte transcription-translation reactions and (A) left untreated or (B) treated with RNase. The crude lysates were then incubated with bacterially expressed MDM2 protein and MDM2 protein (see Fig. 1), and after 1 h, p53 protein was immunoprecipitated with PAb421. The immunoprecipitates were immunoblotted and probed for the presence of MDM2 protein. The arrow marks the position of MDM2 protein bound preferentially to p53F270A (A, lane 5).

The half-lives of the 1) p53F270A mutant protein; 2) p53F19A/F270A double mutant protein; 3) wild-type p53 protein; 4) endogenous MDM2 protein; and 5) transfected MDM2 protein were examined to determine whether the degradation rate of the hyper-ubiquitinated p53F270A mutant protein was MDM2-dependent and whether it differed from wild-type p53 (Fig. 13). Following transfection of wild-type p53 (Fig. 13A, lanes 1–7); wild-type p53 and MDM2 (Fig. 13A, lanes 8–14); p53F270A (Fig. 13B, lanes 1–7); p53F270A and MDM2 (Fig. 13B, lanes 8–14); p53F19A/F270A (Fig. 13C, lanes 1–7); and p53F19A/F270A and MDM2 (Fig. 13C, lanes 8–14); cycloheximide was added for times as indicated from 30 min to 8 h (Fig. 13, lane 2–7 and 9–14). Membranes were blotted with either an anti-p53 monoclonal antibody (DO-1) or an anti-MDM2 monoclonal antibody (2A10), and the steady-state levels of p53 protein (Fig. 13, A–C) and MDM2 protein (Fig. 13, D–F) were examined.

Three major points will be discussed. First, transfection of wild-type p53 (Fig. 13A) induces endogenous MDM2 protein accumulation (Fig. 13D, lane 1) and the addition of cycloheximide results in rapid degradation of the endogenous MDM2 (Fig. 13D, lanes 2–4 versus lane 1). This endogenous MDM2 protein appears incapable of promoting the ubiquitination of transfected wild-type p53 protein. The transfected MDM2 results in ubiquitination of wild-type p53 (Fig. 13A, lane 8 versus lane 1) and the addition of cycloheximide results in the degradation of the ubiquitinated p53 (Fig. 13A, lanes 9–14) that mirrors the degradation of transfected MDM2 (Fig. 13D, lanes 9–14). The non-ubiquitinated p53 protein is not apparently degraded under these conditions (Fig. 13A, lanes 9–14), consistent with the fact that ubiquitination is required to target p53 for degradation. Thus, the MDM2-dependent ubiquitination of p53 by the transfected MDM2 gene is rate-limiting and dependent upon the half-life of MDM2.

Second, transfection of the p53F270A mutant protein resulting in its hyper-ubiquitination (Fig. 13B, lane 1) only marginally induces endogenous MDM2 protein accumulation (Fig. 13E, lane 1), consistent with its lower specific activity in reporter assays (Fig. 10). However, the addition of cycloheximide does not result in the rapid degradation of hyper-ubiquitinated p53F270A mutant protein (Fig. 13B, lanes 2–7) under conditions where the endogenous MDM2 was degraded (Fig. 13E, lanes 2–4). By contrast, the co-transfection of MDM2 with the p53F270A mutant switched the ubiquitination pattern of the p53F270A mutant protein (Fig. 13B, lane 8 versus lane 1), and the addition of cycloheximide resulted in degradation of the ubiquitinated p53F270A mutant protein (Fig. 13B, lanes 9–14) that mirrored the degradation of transfected MDM2 protein (Fig. 13E, lanes 8–14). Additionally, the very high molecular species of p53F270A that actually increase in the presence of cycloheximide (Fig. 13B, lanes 2–7 versus lane 1) were absent after transfection of MDM2 (Fig. 13B, lanes 9–14). These data indicate that the ubiquitination of the p53F270A mutant protein is being carried by a distinct mechanism in the presence of transfected or endogenous MDM2. Consistent with this, the use of His-tagged ubiquitin in a transfection assay does not yield any hyper-ubiquitination of the p53F270A mutant protein (data not shown), suggesting that endogenous pools of ubiquitin are specifically recruited onto the p53F270A mutant protein.

Lastly, despite the fact that the ubiquitinated p53F270A mutant protein has a relatively long half-life (Fig. 13B, lanes 2–7) under conditions where MDM2 is not transfected, the reaction appears MDM2-dependent, because the addition of the Ala19 mutation into the p53F270A mutant protein prevents its hyper-ubiquitination (Fig. 13C, lanes 1–7 and Fig. 11). Furthermore, the addition of cycloheximide does not alter the immunoreactive p53F19A/F270A double-mutant protein bands that remain (Fig. 13C, lanes 2–7 versus lane 1) nor can MDM2 promote the ubiquitination of the p53F19A/F270A double-mutant protein (Fig. 13C, lanes 8–14) under conditions where transfected MDM2 has the same half-life as in other transfections (Fig. 13F, lanes 8–14 versus Fig. 13, D and E, lanes 8–14). Together, these data indicate that the hyper-ubiquitination of the p53F270A mutant protein is MDM2-dependent, but because the half-life of the ubiquitinated p53F270A mutant protein is different depending upon whether endogenous or exogenous MDM2 is present, these data point to a novel assay to dissect out physiologically relevant regulatory steps that affect the rate of p53-ubiquitination by MDM2.

DISCUSSION

P53 protein is a conformationally dynamic allosterically regulated homo-tetramer that has multiple binding sites for regulatory protein co-factors. As such, p53 protein forms that basis for a model oligomeric protein that can give insights into basic mechanisms of protein self-assembly, protein kinase function, transcription, and polyubiquitination mechanisms. P53 protein contains independent sub-domains that direct sequence-specific DNA binding, nonspecific DNA binding, MDM2 or p300 binding, protein kinase binding, and homo-tetramerization. When each domain is linked to the full-length protein, intradomain or allosteric regulation of p53-dependent activity can be observed. For example, the polyproline domain in the N terminus of p53 negatively regulates MDM2 protein binding (29), whereas both the C- and N-terminal domains of p53 can regulate allosterically sequence-specific DNA binding (8, 37–40).
MDM2 protein negatively regulates the tumor suppressor activity of p53 by a variety of overlapping mechanisms. The binding of MDM2 to the N-terminal transactivation domain of p53 can block p53-dependent transcription through steric mechanisms (41, 42). MDM2 protein also normally promotes the polyubiquitination of p53 (43, 44), by which mechanism MDM2 can modulate the steady-state levels of p53 protein. For example, under some conditions, the ubiquitination is associated with rapid degradation of p53 (45). However, polyubiquitination of endogenous p53 also increases after certain types of DNA damage in normal human fibroblasts, indicating that ubiquitin modification does not always trigger the immediate turnover of p53 (46). Nevertheless, the control of p53’s degradation can involve the cytoplasmic transport of p53 protein by MDM2 where p53 is degraded by the proteasome pathway. By contrast to the inhibitory affects of MDM2 on p53 protein levels, MDM2 protein can stimulate TAFII250-dependent exonuclease activity of p53 protein (47, 48) and MDM2 can modulate E2F-dependent activity (64).

The regulation of MDM2 and p53 polypeptide function has been facilitated historically by first dissecting the proteins into individual functional domains. MDM2 protein contains several independent subdomains that either bind to the N-terminal BOX-I domain of p53, to the N terminus of the tumor suppressor p14AGFP, to zinc, or to RNA. This current study addresses the intradomain or allosteric regulation of full-length MDM2 protein and evidence that ligand binding by MDM2 protein switches its affinity and/or specificity from the BOX-I domain of p53 to the conformationally responsive loop flanked by the BOX-IV and BOX-V domains. Three pieces of biochemical data have suggested previously that the MDM2 protein interface with tetrameric forms of p53 involves determinants outside the primary N-terminal BOX-I domain of p53. The first was the observation that MDM2 does not bind to mutant forms of p53 with an altered core domain conformation (25, 49). The second being the observation that full-length MDM2 protein does not bind to p53-DNA complexes in vitro, whereas the truncated N-terminal domain of MDM2 can bind stably to such p53-DNA complexes (18, 25). The final being the observation that MDM2-DNA complexes can bind to p53 protein lacking the N-terminal BOX-I domain (25). The determinants within the p53 tetramer that modulate MDM2 protein binding to the BOX-I domain or that stabilize MDM2 protein binding to sites outside the BOX-I domain were undefined prior to the studies described in this report.

The first protein that was shown to bind to p53 was the SV40 T-antigen (50, 51), although details about the structure of this complex are lacking. This interaction results in the inactivation of p53 function, and the binding occurs within the central domain of the p53 tetramer. Point mutations that unfold the p53 tetramer destabilize the p53-T-antigen complex suggesting a non-linear binding interaction exists between the two proteins (52). Additionally, classically purified T-antigen does not bind to p53 tetramers unless T-antigen is reconstituted with metal ions like zinc (53). The purification of T-antigen results in the loss of the activating metal ion and the re-binding of zinc induces a pronounced conformational change in T-antigen that restores its binding interface with p53 (53). A cellular protein that shares similar features to viral T-antigen is the p53 binding protein BP2. This latter protein was identified in a yeast-two hybrid screen that attempted to define cellular proteins that interact with wild-type but not mutant forms of p53 (54). Like T-antigen, BP2 interacts with p53 in the central domain and certain classes of mutations that can disrupt the p53 tetramer prevent stable binding of BP2 (52). Thus, T-antigen...
MDM2 protein is composed of at least four independent functional domains that in isolation bind to RNA, zinc, ARF14, and the BOX-I domain of p53. We have set out in this report to develop a quantitative biochemical assay that can be used to begin to dissect interdomain or allosteric communication between these domains of MDM2. Three highlights are as follows: 1) MDM2 protein changes its conformation when bound to RNA; 2) this conformational change reduces MDM2 affinity with the primary peptide binding site in the N-terminal domain of p53; 3) this conformational change switches its specificity for a distinct peptide motif in the core domain of p53, pRb, and RNP-U. To our knowledge, this is the first evidence of a protein in a ligand-free and ligand-bound state switching specificity for a target protein and underscores the dynamic nature of MDM2 protein conformation control. It is not yet clear how ligand binding by MDM2 into possible permutations (i.e. MDM2-p14ARF, MDM2-RNA, MDM2-zinc, MDM2-RNA/zinc, etc.) is modulated in cells due to the difficulty in quantitating such MDM2-ligand complexes in living cells. As such it is not yet clear how RNA binding to MDM2 will regulate p53 protein transactivation, polyubiquitination, nuclear export, or degradation. However, these data demonstrate the dynamic nature of a hetero-oligomeric protein-protein interaction where multiple ligand binding sites can direct the docking site specificity and identify an important MDM2 ligand to study for further insights in the p53 pathway. For example, although the homopolyribonucleotide polyG was used as the RNA ligand that binds MDM2, the steady-state levels and accessibility of classes of cellular RNA that can bind to MDM2, like L5 ribosomal protein and its associated 5 S ribosomal RNA (16) or 28 S ribosomal RNA (55), will presumably regulate the pools of MDM2 in a cell that prefer to bind to the BOX-I domain of p53 or to the S9–S10 linker region of p53. In addition, because the RNA binding RING finger domain is required for p53 transport, the MDM2-28 S RNA complex may, for example, be an important mediator of p53 regulation in vivo (55). Degradation of 28 S rRNA occurs during apoptosis in vivo (56). If this degradation destabilizes the MDM2-RNA complex, this may reduce the ability of MDM2 to bind to and inhibit the activity of p53 protein, thus freeing p53 to induce cell death. Our initial data suggest that a significant pool of MDM2-RNA complexes may exist in vivo, based on the observation that the S9–S10 linker mutant p53 proteins are expressed at higher steady-state levels than wild-type p53 protein (data not shown) and that they exhibit increases in MDM2-dependent ubiquitination in cells (Figs. 9 and 13). More quantitative cell-based assays may reveal subtle changes in the levels of wild-type p53 and the S9–S10 linker p53 protein mutants, providing an indirect measure of the MDM2-RNA isofrom and ligand-free forms of MDM2 in cells. Because the RNA-bound form of MDM2 reveals a new peptide binding motif that is present in other proteins like snRNP-U, a protein complex involved in the assembly and/or export of ribosomal RNA particles (57), these data suggest first that the amount of RNA complexed to MDM2 may modulate the stability of other cellular proteins and second that therapeutic agents may be developed that discriminate between the ligand-free and RNA-bound forms of MDM2.

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