Regulation of the Accumulation and Function of p53 by Phosphorylation of Two Residues within the Domain That Binds to Mdm2*

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The function and stability of the tumor suppressor p53 are tightly controlled by the negative regulator mouse double minute 2 (Mdm2), which binds to p53, blocking DNA binding and targeting p53 for proteosome-mediated degradation. Following DNA damage or cellular stress, p53 is phosphorylated within the Mdm2 binding domain on threonine 18 and serine 20. To analyze the roles of these phosphorylation events, residues 18 and 20 were mutated to alanines. Transient transfection into p53-null cells demonstrated that the T18A protein can be expressed stably, but the S20A protein is very unstable, precluding further analysis. When expressed stably at low basal levels in p53-null human fibroblasts or fibrosarcoma cells, the T18A mutant accumulated 5–10-fold less well than wild-type p53 following exposure to UV. Analysis of p53-dependent transcription following UV revealed that the phosphorylation of threonine 18 is required for transactivation of the p21, Hdm2 (the human ortholog of Mdm2), and GADD45 genes. The phosphorylation of serine 33, another early event following DNA damage, is not required for p53 accumulation or p53-dependent transactivation following UV.

The p53 tumor suppressor is a transcription factor that plays a critical role in maintaining genomic stability by mediating cellular growth arrest, DNA repair, and apoptosis after DNA damage. Loss of p53 function increases the potential for cell death and life and death be tightly regulated. Control of p53 function is enforced, in large part, by its negative regulator mouse double minute 2 (Mdm2) or Hdm2 in human cells.

p53 and Mdm2 comprise a feedback loop. Mdm2 binds to the N-terminal domain of p53 (amino acids 17–27), blocking its transactivation function. Because Mdm2 is an E3 ubiquitin ligase, it also modifies p53 and targets it for proteosome-mediated degradation (4–6). p53, in turn, is a transcription factor for the Mdm2 gene (7). Following DNA damage, the p53-Mdm2 interaction is lost, and the p53 protein accumulates and binds to DNA (8). The properties of human tumors and Mdm2-null mice exemplify the importance of this regulatory loop. Mdm2-null mice do not survive because of overexpression of p53, but Mdm2-null, p53-null mice do survive (9). Mdm2 was first identified as a protein overexpressed in a mouse tumor cell line carrying an amplification of the gene in double minute chromosomes (10). Hdm2 has been found to be amplified in human tumors (11). In these cases, overexpression of Mdm2 or Hdm2 depletes cellular p53.

In unstrained cells, p53 is present at a low basal level in a complex with Mdm2 (8). Following DNA damage, the human p53 N-terminal region is phosphorylated on serines 6, 9, 15, 20, 33, and 37 and threonine 18. The association with Mdm2 is lost (8, 12–17). These post-translational modifications also enable p53 to bind to DNA and transactivate its target genes (reviewed in Ref. 1). Mdm2 binds to p53 within a region spanning amino acids 17–27 (18). Threonine 18 and serine 20 are the only p53 phosphorylation sites within the p53-Mdm2 interaction domain; therefore, they are the most likely to regulate the p53-Mdm2 interaction. Consistent with this idea, p53-derived peptides phosphorylated on threonine 18 or serine 20, but not on serines 15, 33, or 37, showed reduced affinity for Mdm2 (13, 19–21).

Threonine 18 is phosphorylated in vitro by casein kinase I (CKI) (13, 22). The role of CKI in the DNA damage response is not known. However, CKI recognition sites are phosphorylated more efficiently when a negative charge is present three residues away in the direction of the N terminus, creating a recognition site for this ubiquitously expressed kinase (reviewed in Ref. 23). For example, serine 15, which is phosphorylated by stress-activated kinases such as DNA-PKcs, ATM, and ATR, is three residues away from threonine 18. The phosphorylation of serine 15 may make threonine 18 more susceptible to phosphorylation by CKI (24–29). In support of this idea, efficient phosphorylation of threonine 18 by CKI was observed only after serine 15 had been phosphorylated (13, 22).

Serine 20 is phosphorylated in vitro by Chk1 and Chk2, DNA damage-responsive kinases that lie downstream of ATM, which is activated by double-strand breaks (30–33). Mutation of serine 20 reduces the stability of p53 after DNA damage (34). Furthermore, Chk2 is a tumor suppressor gene because germ-line mutation of Chk2 results in Li-Fraumeni Syndrome (LFS), a highly penetrant cancer predisposition syndrome usually caused by germ line mutations of p53 (35, 36). Mutations in Chk2 have been identified in human tumors, and p53 is not stabilized in cells from Chk2-null mice following DNA damage (37, 38). Taken together, this evidence suggests a critical role...
for Chk2 in signaling to p53, probably through the phosphorylation of serine 20.

Use of phosphospecific antibodies has demonstrated that both threonine 18 and serine 20 are phosphorylated after DNA damage, suggesting a role for these sites in regulating p53-Mdm2 association and therefore, in p53 protein stability (14, 24, 34, 39). Phosphorylation of serine 20 after exposure of cells to ionizing radiation (IR) or UV correlates with the timing of p53 protein accumulation, which occurs 1–2 h after IR and 4 h after UV (13, 14). The timing of threonine 18 phosphorylation has not been studied as carefully, although one report does show phosphorylation of this site 2 h after IR (13). Thus, the phosphorylations of threonine 18 and serine 20 are relatively early events following DNA damage and are likely to play a role in the dissociation of p53 from Mdm2.

Serine 33 is also phosphorylated very soon after DNA is damaged, within 1 h following irradiation with UV or IR (14, 15, 39). Serine 33 is phosphorylated by Cdk-activating kinase (CAK), a component of the RNA polymerase II holoenzyme (40). This interaction could provide a signal to p53 from RNA polymerase that has been stalled by polymerase poisons or bulky DNA lesions.

We have previously restored wild-type and phosphorylation-site mutant p53 cDNAs to p53-null fibroblasts by using a tetracycline-regulated system (41). This system allowed us to express p53 at low basal levels in cells with a normal p53 response and to examine the properties of these mutant proteins. In the current study, we examine the effect of mutating additional residues that are phosphorylated soon after DNA-damaging events on p53 accumulation and p53-dependent transactivation following DNA damage.

MATERIALS AND METHODS

Constructs—pTO and pTA.hyg were previously reported (42). pTO.neo and pTO.p53 wt.neo were previously described (41). pTO.p53.S20A.neo and pTO.p53.S33A.neo were generated by PCR-SOEing (41) using the S20A mutagenic forward primer, 5′-GGAAACTGGTCCAGGAAGCATTTTC-3′; S33A forward mutagenic primer, 5′-CCATAGGCTCTGCAAATGTTTCC-3′; reverse mutagenic primer, 5′-GGACGGGGGGCCAGAACGTTG-3′; and the reverse primer for amplifying pTO.p53.neo, using the long forward primer T18A.L-F (5′-GTCTATTGCGACTCAGCATGACGTCAGATCCTAGCGTCGAGCC-3′). The T18A mutation was created by SOEing (41) using the S20A mutagenic forward primer, 5′-GGACGGGGGGCCAGAACGTTG-3′. pTO.p53.T18A-R.neo was generated by PCR-amplifying pTO.p53.neo, using the T18A.L-F forward mutagenic primer, 5′-CCATAGGCTCTGCAAATGTTTCC-3′, and reverse mutagenic primer, 5′-GGACGGGGGGCCAGAACGTTG-3′. The PCR product was gel-purified, digested with Ncol, and ligated into pBS.p53 and then pTO.neo as previously described (41). The resulting construct, pTO.p53.T18A-P.neo and all previously described pTO.p53 constructs contain a proline residue at amino acid 72. pTO.p53.T18A-R.neo was constructed by removing the p53 Ncol N-terminal fragment from p53-18APcB6+ (a gift from Margaret Ashcroft, National Institutes of Health, Bethesda, MD) and ligating it into pBS.p53 and pTO.neo. This construct contains an arginine residue at amino acid 72. All others have a proline residue at amino acid 72. Selection of stable clones was done with G418 (400 μg/ml active). Clones screened by Western transfer for p53 expression were prepared by first removing tetracycline for 24 h, then washing the cells with adenosine (ADR) (200 ng/ml) for an additional 24 h to increase the levels of p53. Clones Mwt-3, M18-2R, M18-3R, M33-2, and M33-3 had more p53 protein than Mwt-2; therefore, they were used in the presence of tetracycline (0.01 μg/ml) to normalize the basal p53 levels to that of Mwt-2 cells. The 3–7 cells were derived from HT1080 cells through a mutagenesis strategy described in Agarwal et al. (45) and express 99% less p53 mRNA and p53 protein than parental HT1080 cells. The 3–7.pTA cells were generated by calcium phosphate-mediated transfection of 3-7 cells with pTA.hygro (described in Ref. 42). Stable clones were selected in 250 μg/ml hygromycin. Because the pTA protein is required for pTO promoter function, positive transfectants were identified by their capability to express luciferase. Following calcium phosphate transfection of pTO.luciferase, Luciferase expression was assayed with the Promega assay system. SV2−βgal was co-transfected as a control. A single 3–7.pTA clone, selected for an intermediate level of pTA expression, was chosen as the parental 3–7.pTA line. Hwt, H18, and H33 clones were generated by transfection of 3–7.pTA cells with pTO.p53.neo, pTO.p53.T18A-P.neo, or pTO.p53.S20A.neo in the presence of hygromycin and 1 μg/ml tetracycline. Stable clones were selected in 600 μg/ml (active) G418 and screened for p53 expression by Western analysis. Four separate Hwt, H18, or H33 clones expressing similar low p53 basal levels were identified. Clones were maintained individually. For each experiment, Hwt, H18, or H33 cells were counted using a hemocytometer, pooled in equal numbers, and plated for 24 h prior to treatment.

Western Analyses—Western transfers onto polyvinylidene difluoride (PVDF) membranes (Millipore) were performed with whole cell extracts after separation by SDS-polyacrylamide gel electrophoresis (10% acrylamide). Protein concentrations of lysates were determined by the Bradford method (Bio-Rad), and equal quantities of protein were loaded for each sample. To detect p53, the cells were lysed in 20 mM Tris-hydrochloride, pH 7.4; 1% Nonidet P-40; 150 mM NaCl; 5 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 10 μg/ml apro tin; 25 μg/ml leupeptin; and 1 μg/ml pepstatin A. The membranes were probed with the DO-1 monoclonal antibody to p53 (Santa Cruz Biotechnology), which was detected with a goat anti-mouse antibody conjugated to horseradish peroxidase (Bio-Rad) using enhanced chemiluminescence (Du pont Pharmaceuticals). To quantify the levels of p53, the membranes were analyzed by using enhanced chemiluminescence (Du Pont Pharmaceuticals) and read using a StormImager (Molecular Dynamics). The results were analyzed with ImageQuant software. Serine 15 phosphorylation was detected using phospho-p53 (Ser15) antibody (Perkin Elmer Life Sciences).

Cellular Localization with UV and Treatment with Adriamycin—All treatments were performed 24 h after removal of tetracycline. The UV dose in all cases was measured using a Traceable Ultra Violet Light Meter (Control Company). The optimal dose for each cell type was the dose that gave the highest level of p53 accumulation without down-regulating p53-responsive genes. MHAD041-derived clones were treated with 25 J/m², and HT1080-derived clones were treated with 20 J/m². UV irradiation was performed after removal of most of the culture medium. The optimal adriamycin dose was determined by a dose response curve. Maximal p53 accumulation was achieved at 200 ng/ml medium in MAHA041-derived cells and 300 ng/ml medium in HT1080-derived cells.

Statistical Analysis—An estimate of the mean for each individual clone was calculated as best linear unbiased estimates, where individual clones were considered to be random effects and each mean was regressed toward the overall mean for each clone type (46). The overall mean for each type of clone was estimated as a least squares mean, with individual clones considered to be random effects. Calculated p-values among clone types were adjusted for multiple comparisons using the Tukey-Kramer method to maintain an overall 0.05 significance level for each hypothesis (47). Standard errors for each clone type and individual clone were calculated as the square root of the variance of the mean, which was a function of the within- and between-clone variability. Analyses were done using Mixed Procedure (SAS Statistical Software).

RESULTS

Expression of the T18A and S20A Mutant Proteins—To examine the importance of threonine 18 or serine 20 in regulating p53 stability and function, pTO.p53wt, pTO.p53.T18A-P, and pTO.p53.S20A were transfected transiently into p53-null MAHA041 cells. Western analysis revealed that, although the wild-type and T18A mutant proteins were expressed at high levels, the S20A mutant protein was nearly undetectable (Fig.
Roles of the p53 Phosphorylation Sites Thr<sup>18</sup> and Ser<sup>20</sup>

**Fig. 1.** Transient expression of wild-type, T18A, and S20A p53 proteins and mRNAs. 041/pTA cells were transfected transiently with pTO-p53wt, pTO-p53T18A, or pTO-p53S20A in the absence of tetracycline. The cells were divided and analyzed for p53 expression 36 h after transfection by either the Western (top) or Northern (bottom) procedure.

1). Northern analysis revealed high levels of all three mRNAs, indicating that it is the S20A protein and not the S20A mRNA that is unstable (Fig. 1). Consistent with the data from transient transfections, clones stably expressing the S20A protein could not be generated. These results indicate that serine 20 is required for basal stability of the p53 protein.

**Accumulation of T18A after UV**—We have used a tetracycline-regulated system to express wild-type and phosphorylation site mutant p53 proteins at low basal levels in MDAH041 fibroblasts (41). Expression of relatively high levels of wild-type p53 in these cells results in the transactivation of downstream genes and in growth arrest (44). Six independent 041-derived clones stably expressing T18A were isolated. Clones M18-1P and M18-2P have a proline residue at position 72, whereas M18-1R through M18-4R have an arginine residue at this position. All other wild-type and mutant clones have a proline at amino acid 72. This naturally occurring polymorphism at position 72 does not affect the accumulation of p53 after DNA damage (see below). The accumulation of the T18A mutant protein in response to UV was impaired compared with that of the wild-type clone Mwt3 (Fig. 2A). To quantify the difference between the accumulation of these two proteins, the levels of p53 were measured by chemiluminescence 18 h after irradiation with UV (Fig. 2B). For each clone, the mean percentage increase and the standard error were determined as described in “Materials and Methods.” The average percentage increase for all T18A clones was determined and compared with the wild-type average. Analysis of variance for pairwise comparisons of each type of clone indicated that the accumulation of the protein in M18 clones (average 87%) was significantly lower than in wild-type clones (average 460%; p < 0.001). Therefore, threonine 18 is required for the p53 protein to accumulate efficiently following irradiation with UV in MDAH041 cells.

A second p53-null cell line was also utilized. Following mutagenesis (45), the 3-7 clone was isolated from HT1080 fibrosarcoma cells, which express high levels of wild-type p53. p53 mRNA and protein expression are reduced by 99% in 3-7 cells (data not shown). The levels of exogenous p53 must be carefully regulated by mitogen-activated protein kinase (MAPK) expression to avoid p53 effects. The 3-7 clone was used and data were subsequently obtained.

**Expression of p21, GADD45, or Hdm2 in Response to Irradiation of T18A or S33A with UV**—To determine whether phosphorylation of threonine 18 is required for p53-dependent transactivation, MDAH041, Mwt, and M18 clones were irradiated with UV and p53-responsive mRNAs were analyzed 0, 8, 16, and 24 h later. In all clones expressing wild-type p53, the levels of Hdm2 (the human ortholog of Mdm2), p21 and GADD45 mRNAs increased following irradiation with UV as was previously observed (41). However, the induction of p21, GADD45, and Hdm2 was significantly lower in T18A-expressing clones than in wild-type clones, indicating that threonine 18 is required for efficient accumulation of p53 after UV damage. These results could not be quantified by StormImager analysis because of the relatively weak phosphospecific antibody signal.

**Accumulation of S33A after UV**—Four MDAH041-derived clones stably expressing low basal levels of the S33A mutant protein (M33 clones) were isolated and compared with Mwt clones for p53 accumulation following DNA damage. Four 3-7-derived clones expressing the S33A mutant proteins (H33 clones) were also isolated, pooled, and compared with pooled Hwt clones. p53 in Mwt clones and M33 clones accumulated similarly for 24 h following UV (Fig. 3A). The phosphorylation of serine 15 was normal in M33 clones compared with Mwt clones. p53 accumulation was quantified by chemifluorescence 18 h after irradiation with UV in four independent clones. Analysis of variance for pairwise comparisons between clone types revealed no significant difference between wild-type and mutant clones (Fig. 3B). Similarly, there was no difference in accumulation or serine 15 phosphorylation between wild-type and S33A mutant proteins in pooled 3-7 clones following irradiation with UV (Fig. 3C). Because serine 33 is phosphorylated very soon after irradiation with UV (14, 39), the accumulation of p53 in Mwt and M33 clones 1, 2, and 3 h and Hwt and H33 pools 30, 60, and 90 min after exposure to UV was examined. The accumulation and serine 15 phosphorylation of the wild-type and S33A proteins were similar in both cell systems (Fig. 3D).

**Accumulation of T18A Following Adriamycin**—Mwt, M18, and M33 clones were treated with 200 ng/ml adriamycin, and the accumulation of p53 was compared with Mwt clones over a 24-h time course. The accumulation of p53 in all three was similar (data not shown). The phosphorylation of serine 15 was observed in wild-type, M18, and M33 clones (Fig. 2C and data not shown). Quantitative Western analysis 18 h after ADR treatment confirmed that there was no difference in protein accumulation in Mwt, M8, and M33 clones (Fig. 4A). The overall response to ADR in 041 cells is weak compared with the response to UV (an increase of 86% compared with 460%). When Hwt clones were treated with 300 ng/ml ADR, the response was not only more robust than observed in 041 cells (a 400% increase) but also much closer to the response observed after UV treatment. The H18 clones accumulated less protein after ADR treatment than did the Hwt clones (Fig. 4B). The phosphorylation of serine 15 was also examined using the Ser<sup>P</sup>15 antibody. Hwt clones showed high levels of serine 15 phosphorylation after ADR treatment, whereas the H18 clones showed a lower level, probably because of the lower levels of p53. The Mwt/M18 results allow a delineation of the ADR and UV signaling pathways to p53, and the Hwt/H18 results demonstrate a requirement for threonine 18 for efficient accumulation of p53 after ADR treatment. Comparison of Hwt and H33 pools yielded the same result as comparison of Mwt and M33 clones; serine 33 phosphorylation was not required for p53 to accumulate or for serine 15 phosphorylation after ADR treatment (Fig. 4C).

**Expression of p21, GADD45, or Hdm2 in Response to Irradiation of T18A or S33A with UV**—Four MDAH041-derived clones stably expressing low basal levels of the S33A mutant protein (M33 clones) were isolated and compared with Mwt clones for p53 accumulation following DNA damage. Four 3-7-derived clones expressing the S33A mutant proteins (H33 clones) were also isolated, pooled, and compared with pooled Hwt clones. p53 in Mwt clones and M33 clones accumulated similarly for 24 h following UV (Fig. 3A). The phosphorylation of serine 15 was normal in M33 clones compared with Mwt clones. p53 accumulation was quantified by chemifluorescence 18 h after irradiation with UV in four independent clones. Analysis of variance for pairwise comparisons between clone types revealed no significant difference between wild-type and mutant clones (Fig. 3B). Similarly, there was no difference in accumulation or serine 15 phosphorylation between wild-type and S33A mutant proteins in pooled 3-7 clones following irradiation with UV (Fig. 3C). Because serine 33 is phosphorylated very soon after irradiation with UV (14, 39), the accumulation of p53 in Mwt and M33 clones 1, 2, and 3 h and Hwt and H33 pools 30, 60, and 90 min after exposure to UV was examined. The accumulation and serine 15 phosphorylation of the wild-type and S33A proteins were similar in both cell systems (Fig. 3D).

**Accumulation of T18A Following Adriamycin**—Mwt, M18, and M33 clones were treated with 200 ng/ml adriamycin, and the accumulation of p53 was compared with Mwt clones over a 24-h time course. The accumulation of p53 in all three was similar (data not shown). The phosphorylation of serine 15 was observed in wild-type, M18, and M33 clones (Fig. 2C and data not shown). Quantitative Western analysis 18 h after ADR treatment confirmed that there was no difference in protein accumulation in Mwt, M8, and M33 clones (Fig. 4A). The overall response to ADR in 041 cells is weak compared with the response to UV (an increase of 86% compared with 460%). When Hwt clones were treated with 300 ng/ml ADR, the response was not only more robust than observed in 041 cells (a 400% increase) but also much closer to the response observed after UV treatment. The H18 clones accumulated less protein after ADR treatment than did the Hwt clones (Fig. 4B). The phosphorylation of serine 15 was also examined using the Ser<sup>P</sup>15 antibody. Hwt clones showed high levels of serine 15 phosphorylation after ADR treatment, whereas the H18 clones showed a lower level, probably because of the lower levels of p53. The Mwt/M18 results allow a delineation of the ADR and UV signaling pathways to p53, and the Hwt/H18 results demonstrate a requirement for threonine 18 for efficient accumulation of p53 after ADR treatment. Comparison of Hwt and H33 pools yielded the same result as comparison of Mwt and M33 clones; serine 33 phosphorylation was not required for p53 to accumulate or for serine 15 phosphorylation after ADR treatment (Fig. 4C).
Hdm2, and GADD45 mRNA in M18 clones was nearly identical to the induction observed in p53-null MDAH041 cells (Fig. 5A). In the absence of p53 or in the presence of T18A, there was little or no induction of p53 target genes, despite a slight increase in the mutant protein in M18 clones after irradiation with UV. We previously demonstrated that S15A/S37A mutant p53 is deficient in accumulation after irradiation with UV. However, it does transactivate Hdm2, p21, and GADD45 after irradiation with UV, but less well than wild-type p53 (Fig. 6, Ref. 41). Although accumulation of the S15A, S37A, S15A/S37A, and T18A mutant proteins in MDAH041-derived clones was identical (data not shown), the defect in clones expressing T18A was much more dramatic, indicating that threonine 18 is required for p53-dependent transactivation after irradiation with UV.

Phosphorylation of threonine 18 may be required for all p53-dependent transactivation. This possibility was examined by overexpressing wild-type T18A and, as a negative control, S20A proteins in 041.pTA cells. p53 and p21 proteins were analyzed by the Western procedure 36 h after transfection (Fig. 5B). As expected, high levels of wild-type and T18A proteins were observed, but p53 was not detected in untransfected 041.pTA cells or in cells transfected with S20A cDNA. p21 protein levels were induced in the presence of wild-type and T18A mutant p53 proteins compared with MDAH041 and the cells transfected with p53.S20A-transfected cells. These data indicate that, although threonine 18 is required for transactivation of p53 target genes after irradiation with UV, when overexpressed, the T18A mutant protein can drive p53-dependent transactivation.

Using Mwt and M33 clones, the requirement for serine 33 phosphorylation in transactivating p53 target genes was tested. Because the wild-type and S33A proteins accumulated identically after irradiation with UV in both MDAH041 and 3-7 cells, we expected that p53-responsive mRNAs would accumulate identically if this phosphorylation event were not required. The expression of Hdm2, p21, and GADD45 in M33 clones was compared with the Mwt-3 clone at several times after irradiation. A representative experiment is shown in Fig. 5C. There was no difference in the accumulation of downstream mRNAs in M33 clones compared with Mwt clones.

**DISCUSSION**

p53 responds to many types of DNA damage and adverse environmental conditions to drive a cellular response tailored to the specific inducer (1–3). Previously, we expressed wild-type and phosphorylation-site mutant p53 proteins at normal controlled basal levels in p53-null fibroblast cell lines, which have many of the properties of normal fibroblasts, allowing a careful study of protein accumulation following DNA damage (41). These studies demonstrated that the phosphorylation of serines 15 and 37, which lie outside the p53-Mdm2 interaction domain, is required for p53 to accumulate after irradiation with UV. In the current study we have expanded our analysis to three other phosphorylated residues and again, using well-controlled systems, have been able to assign functions to two of these sites.

The instability of the S20A protein can be explained by the location of serine 20 in the p53-Mdm2 interaction domain. An inability to phosphorylate serine 20 probably allows stable
binding of Mdm2 to p53, eliminating even basal expression of p53 protein in MDAH041 and 3-7 cells. The identification of Chk2, which encodes the serine 20 kinase, as a gene mutated in Li-Fraumeni Syndrome supports this idea (35). When Chk2 is not expressed, p53 protein cannot function. However, the almost complete instability we have observed is somewhat different from the results of Chehab et al. (34), who report a lower basal level of human S20A mutant protein compared with wild-type p53 in transiently transfected p53-null mouse embryo fibroblasts. They also report a failure of tagged S20A protein to accumulate from a nearly normal basal level in human U2-OS cells that express endogenous wild-type p53. The lack of even basal S20A expression we observed in MDAH041 and 3-7 cells is much more dramatic and must reflect the integrity of p53-dependent pathways in these cells.

Mutation of threonine 18, but not serine 33, resulted in a significant defect in p53 accumulation after UV treatment. Similar to serine 20, threonine 18 also resides within the p53-Mdm2 interaction domain; however, the latter residue is not required for basal expression of p53, as shown by the result of transient expression of the T18A mutant protein compared with wild-type p53 in transiently transfected p53-null mouse embryo fibroblasts. They also report a failure of tagged S20A protein to accumulate from a nearly normal basal level in human U2-OS cells that express endogenous wild-type p53. The lack of even basal S20A expression we observed in MDAH041 and 3-7 cells is much more dramatic and must reflect the integrity of p53-dependent pathways in these cells.

Mutation of threonine 18, but not serine 33, resulted in a significant defect in p53 accumulation after UV treatment. Similar to serine 20, threonine 18 also resides within the p53-Mdm2 interaction domain; however, the latter residue is not required for basal expression of p53, as shown by the result of transient expression of the T18A mutant protein and isolation of stable clones expressing basal levels of T18A. Threonine 18 is phosphorylated in response to DNA damage, and this phosphorylation could disrupt the interaction of p53 with Mdm2 directly, thus explaining the lack of accumulation of p53 following irradiation with UV in M18 and H18 clones. The phosphorylation of N-terminal peptides of p53 on threonine 18 reduces their affinity for Mdm2 (19, 20).

We demonstrated previously that the phosphorylation of serine 15 is required for p53 to accumulate after irradiation with UV (41). The in vivo effects of serine-to-alanine mutations outside the p53-Mdm2 interaction domain might be explained by the fact that the phosphorylation of serine 15 is required for efficient phosphorylation of N-terminal peptides on threonine 18 (13, 22). By mutating serine 15 we may have disrupted the CKI-dependent phosphorylation of threonine 18. However, there was no difference in the accumulation of the T18A and S15A mutant proteins in MDAH041 cells. This result could indicate an absolute requirement in vivo of serine 15 for threonine 18 phosphorylation. An antibody specific for threonine 18 phosphorylation would allow this hypothesis to be tested, but this reagent is not commercially available.

Our results in two different p53-null cells differ in the responses to ADR. However, these observations support our earlier findings that UV and ADR signal to p53 through separate pathways. These pathways can be delineated in 041 cells (41). The differences between the 041 and 3-7 cell lines have allowed us to draw two important conclusions. First, the generally weak accumulation of wild-type and mutant p53 proteins observed in 041 cells after ADR treatment suggests a possible upstream mutation in this cell line that precludes a normal response to this drug. The strong response of wild-type, but not

**Fig. 3.** Accumulation of S33A p53 after irradiation with UV. A, Mwt and M33 clones were irradiated with UV (25 J/m²), and the amount of p53 protein was analyzed by Western transfer over a 24-h period after treatment (top). Serine 15 phosphorylation was detected by using a phosphospecific antibody (bottom). B, individual Mwt and M33 clones were analyzed in several independent experiments 18 h after irradiation with UV, and the percentage increases in p53 were quantified by using chemiluminescence (left). Individual data points and best linear unbiased estimates of the mean with standard errors are shown for each clone. There was no significant difference between the average percentage increases in p53 protein in Mwt and M33 clones (right). C, Hwt- and H33-pooled clones were irradiated with UV (20 J/m²), and the accumulation (top) and serine 15 phosphorylation (bottom) of p53 were monitored by Western analysis over a 24-h period. D, accumulation (top) and serine 15 phosphorylation (bottom) of p53 in Mwt and M33 clones and pooled Hwt and H33 clones were monitored soon after irradiation with UV.
mutant, p53 after UV treatment demonstrates a separation of the UV and ADR pathways. Secondly, the robust response of wild-type p53 to ADR in 3-7 cells requires threonine 18, suggesting that the phosphorylation of this residue is indispensable for normal protein accumulation after treatment with ADR. The observation that serine 15 is phosphorylated weakly in 041 cells, but strongly in 3-7 cells after treatment with ADR, supports the idea that p53 responds differently to ADR in these two cell types.

Transactivation of p53-responsive genes was studied in MDAH041 cells because they retain many normal cellular responses to p53 (44). In the M18 clones, target genes were expressed at very low levels, virtually identical to the levels in MDAH041 cells lacking p53 after irradiation with UV. Because the T18A protein does not accumulate efficiently after irradiation with UV, we could not determine directly whether T18A is capable of functioning as a transcription factor. By comparing clones expressing T18A and S15/37A, in which p53 accumulation is identically impaired after irradiation with UV, a distinction between a deficiency in accumulation or in transactivation could be made for the T18A protein.

The capability of the T18A protein to drive the transcription of p21 when overexpressed suggests that phosphorylation is required after irradiation with UV to prevent the binding of a negative regulator such as Mdm2. Overexpression would be expected to overwhelm this regulatory interaction. If phosphorylation were required for the binding of a transcription factor such as CBP, overexpression would probably not overcome the need for phosphorylation at this site. The in vivo requirement of threonine 18 for p53-mediated transactivation demonstrates the tight control of p53 function in response to UV-activated pathways, which can be lost when p53 is overexpressed.

We conclude that the phosphorylation of threonine 18, but not serine 33, is required for transactivation of the p53 target genes analyzed in response to irradiation with UV. The trend observed may indicate that threonine 18 must be phosphorylated to relieve the blockade of the p53 transactivation domain by Mdm2. Phosphorylation of other residues, such as serine 15, 33, or 37, may be required for different subsets of genes. For example, the observation that the phosphorylation of serine 15 is required for basal p21 expression demonstrates a specific role for this site (41). Similarly, the phosphorylation of serine 46 is required for transactivation of the AIP1 gene (49). Analyzing the expression of hundreds of p53-responsive genes in cells expressing specific serine- or threonine-to-alanine mutants will determine whether our observations can be generalized to many other transcriptional targets of p53.

No defects in protein accumulation or p53-dependent transactivation were observed in cells expressing S33A. Because we examined only a small subset of p53-dependent functions, it is possible that the phosphorylation of serine 33 is required for functions not tested here. For example, the phosphorylation of serine 33 by Cdk-activating kinase could occur in response to a blockade of RNA polymerase (40). It has been proposed that low doses of UV radiation generate bulky DNA lesions that block RNA polymerase, thus generating a signal to p53 (48, 50). We examined the accumulation of S33A protein after low doses of UV in two different p53-null cells, but did not observe a defect in accumulation. These
results indicate that serine 33 is either not required for the p53 response to bulky DNA lesions, or that low doses of UV radiation generate a different signal to p53.

The expression of p53 at low basal levels in a well-controlled system has allowed us to establish a functional role for its phosphorylation in response to DNA damage. This study, in combination with our previous work, provides compelling evidence that serines 15, 20, and 37, and threonine 18 are important in the regulation of p53 accumulation and function and should be studied further.

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REFERENCES

1. Ljungman, M. (2000) Neoplasia 2, 208–225
2. Prives, C., and Hall, P. A. (1999) J. Pathol. 187, 112–126
3. Agarwal, M. L., Taylor, W. R., Chernov, M. V., Chernova, O. B., and Stark, G. R. (1998) J. Biol. Chem. 273, 1–4
4. Haupt, Y., Maya, R., Kaxaz, A., and Oren, M. (1997) Nature 387, 296–299
5. Honda, R., Tanaka, H., and Yasuda, H. (1997) FEBS Lett. 420, 25–27
6. Kubbhatat, M. H., and Vouuden, K. H. (1997) Mol. Cell. Biol. 17, 460–468
7. Moman, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. (1992) Cell 69, 1237–1245
8. Fuchs, S. Y., Adler, V., Buschmann, T., Wu, X., and Ronai, Z. (1998) Oncogene 17, 2543–2547
9. Jones, S. N., Roe, A. E., Donehower, L. A., and Bradley, A. (1995) Nature 378, 204–208
10. Fakharzadeh, S. S., Trusko, S. P., and George, D. L. (1991) EMBO J. 10, 299–306
11. Bulavin, D. V., Saito, S., Hollander, M. C., Sakaguchi, K., Anderson, C. W., Appella, E., and Fornace, A. J., Jr. (1999) Oncogene 18, 4047–4054
12. Higashimoto, Y., Saito, S., Tong, X. H., Hong, A., Sakaguchi, K., Appella, E., and Anderson, C. W. (1992) Mol. Cell. Biol. 12, 4054–4059
13. Shieh, S. Y., Ahn, J., Tamai, K., Taya, Y., and Prives, C. (2000) Genes Dev. 14, 289–300
14. Shieh, Y. H., Lin, W. S., Lin, W. K., Sim, J., and Haber, D. A. (1999) Science 286, 2526–2531
15. Prives, C., and Hall, P. A. (1999) J. Biol. Chem. 274, 18151–18156
16. Shieh, S. Y., and Prives, C. (1999) EMBO J. 18, 1815–1823
17. Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. (1992) Cell 69, 1237–1245
18. Kussie, P. H., Gore, S., Marenchuk, V., Kolenko, B., Gocke, L., and Pavletich, N. P. (1998) Science 274, 948–953
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