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Damage-mediated Phosphorylation of Human p53 Threonine 18 through a Cascade Mediated by a Casein 1-like Kinase

EFFECT ON Mdm2 BINDING*

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The p53 tumor suppressor protein is stabilized in response to ionizing radiation and accumulates in the nucleus. Stabilization is thought to involve disruption of the interaction between the p53 protein and Mdm2, which targets p53 for degradation. Here we show that the direct association between a p53 N-terminal peptide and Mdm2 is disrupted by phosphorylation of the peptide on Thr^{18} but not by phosphorylation at other N-terminal sites, including Ser^{15} and Ser^{37}. Thr^{18} was phosphorylated *in vitro* by casein kinase (CK1); this process required the prior phosphorylation of Ser^{15}. Thr^{18} was phosphorylated *in vivo* in response to DNA damage, and such phosphorylation required Ser^{15}. Our results suggest that stabilization of p53 after ionizing radiation may result, in part, from an inhibition of Mdm2 binding through a phosphorylation-phosphorylation cascade involving DNA damage-activated phosphorylation of p53 Ser^{15} followed by phosphorylation of Thr^{18}.

The p53 tumor suppressor protein is a critical regulator of cell cycle progression that responds to DNA damage and certain other cellular stresses by arresting cell cycle progression or by inducing apoptosis (1–3). These responses are important for preserving the integrity of the genome of a cell, thus preventing the transformation of a normal cell into a tumor cell. p53 normally is a short-lived protein that is maintained at low levels in unstressed cells. After cells are exposed to DNA-damaging agents, nucleotide depletion, or hypoxia, the p53 protein is transiently stabilized and accumulates in the nucleus. DNA damage also activates p53 as a transcription factor, which in turn induces or represses the transcription of several genes, including *WAF1* and *MDM2*, that regulate cell cycle progression. Both stabilization of the p53 protein and activation of its sequence-specific DNA binding ability are widely believed to be mediated, at least in part, by post-translational modifications to the p53 protein. p53 is phosphorylated on several sites in its N-terminal transactivation domain as well as on several sites in the C-terminal tetramerization/regulatory domain (4), and recent studies have shown that serines 15, 33, and 37 become phosphorylated after cells are exposed either to ionizing radiation (IR)[1] or to UV light (5–7). In undamaged cells, p53 levels are low because the protein is rapidly degraded through a ubiquitin-mediated pathway (8, 9) that is mediated by the Mdm2, a protein that binds to the N terminus of p53 (10, 11) and serves as an ubiquitin ligase (12). Recently, it was reported that incubating p53 with DNA-PK, a protein kinase activated by DNA strand breaks that phosphorylates human p53 *in vitro* on both Ser^{15} and Ser^{37} (13), inhibited the binding of p53 to Mdm2 (5, 12, 14). Thus, phosphorylation of these sites *in vivo* may stabilize p53 by preventing Mdm2 binding.

To test this prediction, we measured the binding constant for peptides corresponding to the N terminus of p53 and a human Mdm2 fragment that binds p53 (11). Surprisingly, phosphorylation of Ser^{15}, of Ser^{37}, or of both residues had only a small effect on the binding constant. Hence, either the inhibition of p53-Mdm2 binding by DNA-PK was not due to its phosphorylation of p53 or the observed effects were an indirect consequence of that phosphorylation. Here, we show that the latter explanation may be correct. We demonstrate that phosphorylation of Thr^{18} of p53 by casein kinase 1 (CK1) or a CK1-like activity is activated by phosphorylation of Ser^{15} and that this phosphorylation can account, in part, for the inhibition of p53 binding by Mdm2 observed after exposing cells to ionizing radiation.

EXPERIMENTAL PROCEDURES

Cells and Cell Transfections—A549 (ATCC CCL-185), a human lung carcinoma cell line that expresses wild-type p53, was obtained from the American Type Culture Collection (Manassas, VA); NCI-H1299 (ATCC CRL-5803), a human lung carcinoma cell line that is null for both p53 alleles (15), was obtained from A. Fornace, Jr. (NCI, National Institutes of Health). Cells were grown to 50–70% confluency in Dulbecco's modified minimal essential medium supplemented with 10% fetal bovine serum and 100 nM glutamine in 150-mm dishes. Cultures were transfected with 10 μg of DNA/plate by the LipofectAMINE Plus procedure as described by the manufacturer (Life Technologies, Inc.). One day

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[1] The abbreviations used are: IR, ionizing radiation; ALLN, *N*-acetyl-Leu-Leu-Nle-CHO; CK1, casein kinase 1; DNA-PK, DNA-activated protein kinase; F_2Pab, (2-amino-4-phosphono)-4,4-difluorobutanoic acid); Fmoc, 9-fluorenylmethoxycarbonyl; MES, 4-morpholineethanesulfonic acid.

after transfection, cultures were exposed to 8 gray by using a Shepherd Mark I ^{137}Cs irradiator or treated with ALLN, a proteasome inhibitor, as described (7). After the times indicated (see Fig. 3), cultures were harvested and processed for immunoprecipitation and immunoblotting.

Phosphorylation-specific p53 Antibodies—Antibodies specific for p53 phosphorylated at Thr[18] or Ser[20] were prepared as described (7). Briefly, the PabSer(P)20 and PabThr(P)18 antibodies were made by immunizing rabbits with the p53 phosphopeptides Ac-15–26(20P)C (*i.e.* Ac-Ser-Gln-Glu-Thr-Phe-Ser(P)-Asp-Leu-Trp-Lys-Leu-Leu-Cys-NH_2) or Ac-13–24(15, 18P)C (*i.e.* Ac-Pro-Leu-Ser(P)-Gln-Glu-Thr(P)-Phe-Ser-Asp-Leu-Trp-Lys-Cys-NH_2) coupled to keyhole limpet hemocyanin, respectively. Phosphorylation site-specific antibodies were affinity purified from the resulting serum using the corresponding phosphorylated peptides attached to SulfoLink (Pierce). In the case of PabThr(P)18, the antibody was depleted of Ser[15](P) cross-reacting activity by also passing the preparation through a column of Ac-p53(13–24)(15P)C attached to SulfoLink. The antibody to p53 phosphorylated at Ser[15] was made by immunizing a rabbit with the peptide human p53(13–19(15F_2Pab))A(13–19(15F_2Pab))AC coupled to keyhole limpet hemocyanin, *i.e.* PL(F_2Pab)QETAPL(F_2Pab)QETAC-KLH. F_2Pab is a phosphoserine mimetic (16) and was incorporated as the BOC-F_2Pab(OEt_2-OH) derivative. Phosphoserine 15-specific antibodies were purified using the natural phosphopeptide (p53(Ac-11–22(15P)Cys)-SulfoLink conjugate.

Synthetic Phosphopeptides—Peptides were synthesized by the solid phase method with Fmoc chemistry using an Applied Biosystems (Foster City, CA) 430A peptide synthesizer. Phosphoserine was incorporated as Fmoc-Ser(PO(OBzl)OH)-OH, and phosphothreonine was incorporated as Fmoc-Thr(PO(OBzl)OH)-OH (Novabiochem, San Diego, CA). Peptide p53(15–29) was labeled with 5-carboxyfluorescein, succinimidyl ester (Pierce) on the resin. Peptides were cleaved from the resin, and side chain protecting groups were removed by incubating in reagent K (trifluoroacetic acid:phenol:thioanisole:H_2O:EDT, 82.5:5:5:5:2.5) for 3 h at room temperature. The p53(1–39) peptides were purified by high pressure liquid chromatography on a pH-stable Vydac C-8 column (Hesperia, CA) with 0.2% hexafluoroacetone-NH_4OH, pH 7.0, acetonitrile. The masses of peptides were confirmed by electrospray ionization mass spectrometry on a Finnigan MAT SSQ 7000 (Finnigan MAT, San Jose, CA).

Measurement of p53-Mdm2 Dissociation Constants—The dissociation constants (K_d) for unlabeled p53 peptides from the Mdm2 fragment were determined by a competition assay with fluorescein-labeled p53(15–29) in 25 mM MES, pH6.8, 100 mM NaCl, 0.5 mM EDTA containing 0.1 mg/ml bovine serum albumin at 4°C (17, 18). Fluorescence anisotropy was measured in a PanVera Beacon 2000 instrument with temperature control (PanVera Corporation, Madison, WI). Binding constants were determined as follows. The binding constant of the labeled peptide was first determined by titrating 50 nM fluorescein-labeled p53(15–29) with increasing amounts of Mdm2(17–125) and measuring the fluorescence anisotropy at each concentration. In separate experiments, 50 nM of fluorescein labeled p53(15–29) was mixed with several different concentrations of unlabeled peptides, and these mixtures were titrated with the Mdm2 fragment. Fluorescence anisotropy was recorded at each concentration.

The binding constant of the fluoresceinated p53(15–29) peptide was determined by fitting to a single site binding equation using either SigmaPlot (SPSS Inc., San Rafael, CA) or a BASIC program written by the authors. For the competition assays, binding isotherms at three concentrations were simultaneously fitted by a global-fit program developed by the authors to the following equations.

$$A_{obs} = A_f + (A_c - A_f)[P^*M]/[P^*]_T \quad \text{(Eq. 1)}$$

$$[P^*M] = K^*[P^*]_T X/(1 + K^* \cdot X) \quad \text{(Eq. 2)}$$

$$X = (((1 + K[P]_T - K[M]_T)^2 + 4 \cdot K[M]_T)^{1/2} - (1 + K[P]_T - K[M]_T))/2K \quad \text{(Eq. 3)}$$

A_f is the anisotropy of the free labeled peptide, A_c is the anisotropy of the labeled peptide-Mdm2 complex, A_{obs} is the observed anisotropy, K^* is the association constant of the labeled peptide with the Mdm2 fragment, K is the association constant of the unlabeled peptide with the Mdm2 fragment, $[P^*]_T$ is the total concentration of labeled peptide, $[P]_T$ is the total concentration of unlabeled competing peptide, $[P^*M]$ is the concentration of the labeled peptide-Mdm2 complex, and $[M]_T$ is the total concentration of Mdm2 fragment. These equations were derived from the Weber average formula (19) and the mass action law, assuming that $[M]_f + [PM] \gg [P^*M]$, where $[M]_f$ is the free Mdm2 fragment concentration and $[PM]$ is the concentration of the unlabeled peptide-Mdm2 fragment complex. Because of the concentration of the labeled peptide used and the affinity constants, such an assumption is fully justified.

Kinase Reactions—The p53(Ac-1–24) peptides at 200 μM were incubated at 30 °C for 30 min with recombinant CK1-δ (New England BioLabs) in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, and 200 μM ATP containing [γ-^{32}P]ATP. The ^{32}P-labeled peptides were separated by chromatography in *n*-butanol:prydine:acetic acid:water (3:2:1:4) on cellulose TLC plates (Merck). A recombinant fusion protein, N47 (20), containing residues 2–47 of human p53, at 38 μM was incubated at 30 °C for 30 min with DNA-PK (Promega) in 50 mM Hepes, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 200 μM ATP, and 10 μg/ml calf thymus DNA. The reactants then were diluted into an equal volume of a CK1-δ reaction as described above, but without peptide, and incubation was continued for an additional 30 min. Phosphoamino acid analysis was performed as described (21).

Detection of p53 Phosphorylation by Western Immunoblotting—Cell extracts were prepared, and Western immunoblots were performed essentially as described (7); however, in place of immunoprecipitation with protein G-Sepharose, p53 was collected by centrifugation with a 1:1 mixture of PAb1801- and DO-1-agarose beads (Santa Cruz Biotechnology, Inc.). 5 μg of each antibody conjugate was used for 1300 μg of extract protein; after incubation for 2 h at 4 °C, the beads were washed five times by centrifugation. To avoid possible interference by the IgG heavy chain, the reducing agent was omitted from the SDS-polyacrylamide gel electrophoresis sample buffer.

RESULTS

Interaction of p53(1–39) and Mdm2(17–125)—To examine directly the effect of N-terminal phosphorylation on the interaction of p53 and Mdm2, we determined the dissociation constants for various phosphorylated p53(1–39) peptides with Mdm2(17–125) by measuring the changes in fluorescence anisotropy of fluorescein-labeled p53(15–29) complexed with Mdm2(17–125) in response to added p53(1–39) (Fig. 1 and Table I). Different amounts of Mdm2 were added to mixtures of the fluorescein-labeled probe and p53(1–39) or p53(1–39) phosphorylated at different positions. The dissociation constant for each peptide with Mdm2 then was determined by fitting the curves using a mathematical model (see "Experimental Procedures"). Unphosphorylated p53(1–39) bound Mdm2 with an apparent dissociation constant of 70 ± 20 nM (Fig. 1*A*), whereas p53(1–39) with Ala-Ala replacing Leu[22]-Trp[23] exhibited no measurable binding (Fig. 1*D*). Surprisingly, p53(1–39) phosphorylated on Ser[15] (Fig. 1*B*), on Ser[37] or on both residues had dissociation constants that were only slightly higher (60–260 nM) on average than the unphosphorylated peptide (Table I). Phosphorylation of the p53 N-terminal peptide on Ser[9], Ser[20], Ser[33], or Ser[15] and Ser[20] also only slightly affected the interaction of p53 and Mdm2. In contrast, phosphorylation of Thr[18] or of Thr[18] and Ser[15] (Fig. 1*C*) dramatically reduced the association (~30–50-fold). Contrary to previous reports, these data suggest that phosphorylation of p53 at Ser[15] and Ser[37] does not directly inhibit formation of the p53-Mdm2 complex; rather, phosphorylation of Thr[18], which stabilizes the association with Mdm2 through hydrogen bonding of the threonine hydroxyl group (11), appears to be crucial.

Phosphorylation of Thr[18] in Vivo in Response to Ionizing Radiation—To explore the question of its phosphorylation, we prepared an antibody that specifically recognizes p53 only when it is phosphorylated on Thr[18]; we also made antibodies that recognize p53 only when it is phosphorylated on Ser[15] or on Ser[20]. The specificities of these antibodies for p53 phosphorylated at the respective sites were confirmed by peptide enzyme-linked immunosorbent assays and/or by peptide dot-blot assays as reported recently for other p53 phosphorylation-specific antibodies (7). Fig. 2 shows the specificity of the p53 phospho-Thr[18] antiserum as determined by enzyme-linked immunosorbent assay. The antiserum recognized the p53(1–39) peptide phosphorylated on Thr[18] alone or on Thr[18] and Ser[15]. It also recognized a p53(Ac-13–24) peptide phosphorylated at

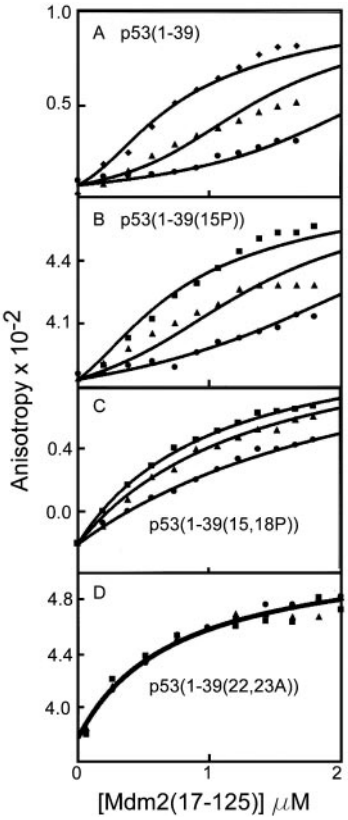

FIG. 1. **Effect of N-terminal phosphorylation on the interaction of p53(1–39) with Mdm2(17–125).** The anisotropy of fluorescein-p53(15–29) was determined as a function of Mdm2(17–125) concentration for three different concentrations of competitor p53(1–39) peptide that was unphosphorylated (*A*), phosphorylated on Ser[15] only (*B*), phosphorylated on Ser[15] and Thr[18] (*C*), or mutated to change both Leu[22] and Try[23] to Ala (*D*), as described under "Experimental Procedures." Competitor peptide concentrations for *A* and *B* were 0.3 μM (♦), 1.0 μM (▲), and 2.0 μM (●); competitor peptide concentrations for C were 1 μM (♦), 2 μM (▲), and 5 μM (●); competitor peptide concentrations for *D* were 5 μM (♦), 10 μM (▲), and 15 μM (●). Data points were determined in triplicate and fitted to a set of equations as described under "Experimental Procedures" to determine the apparent dissociation constant (K_d) for the competitor peptide-Mdm2(17–125) complex. Similar experiments were performed to determine dissociation constants for other peptides described in Table I.

TABLE I
Binding affinity of p53(1–39) peptides with Mdm2(17–125)

Peptide[a]	K_d[b]	±S.D.[c]
	nM	*nM*
1–39	70	20
1–39(9P)	120	60
1–39(15P)	170	20
1–39(18P)	3400	660
1–39(20P)	110	40
1–39(33P)	210	140
1–39(37P)	260	90
1–39(15,18P)	2400	200
1–39(15,20P)	70	20
1–39(15,37P)	60	30
1–39(22,23Ala)[d]	>190,000	NA
p73(1–37)[e]	430	ND

[a] Human p53(1–39) peptides phosphorylated at the site or sites indicated.
[b] The apparent dissociation constants were determined from measurements of the fluorescence anisotropy (see "Experimental Procedures").
[c] Each value is the average of at least three determinations. NA, not applicable; ND, not determined.
[d] Residues Leu[22] and Trp[23] in the p53(1–39) peptide were changed to Ala.
[e] First 37 residues from human p73 (GenBank™ accession number 3414961, MAQSTATSPD GGTTFEHLWS SLEPDSTYFD LPQSSRG).

Thr[18] and Ser[20], although not so well. It did not recognize the unphosphorylated p53(1–39) peptide, the peptide with Ser[15] changed to alanine, the p53(1–39) peptide phosphorylated at Ser[15], the peptide phosphorylated at both Ser[15] and Ser[20], nor several other control peptides; however, the antibody did recognize the p53(1–39) peptide with Ser[15] changed to alanine and Thr[18] phosphorylated (data not shown). These data show that the PAbThr(P)18 antiserum specifically recognizes p53 when phosphorylated on Thr[18] but not on other N-terminal p53 phosphorylation sites; its recognition is not abrogated by phosphorylation at Ser[15] or Ser[20] nor by changing Ser[15] to another amino acid.

Fig. 3 shows that p53 Thr[18] was not detectably phosphorylated in unirradiated A549 cells nor in cells treated with the proteasome inhibitor ALLN, which stabilizes p53 (22), as seen with DO-1, a monoclonal antibody specific for the N terminus of p53. However, after cells were exposed to 8 gray of γ-irradiation, a dramatic, transient phosphorylation of Thr[18] was observed by 45 min that peaked 2 h after exposure and disappeared between 8 and 24 h. Ser[20] also was phosphorylated after IR with similar initial kinetics, but it remained phosphorylated at 24 h. As shown previously (5, 6), phosphorylation of Ser[15] is rapidly induced by IR and was seen within 45 min of exposure; phosphorylation peaked at 2 h and then slowly declined (Fig. 3). Similar to Thr[18], Ser[15], or Ser[20] were not phosphorylated in unirradiated A549 cells nor in cells treated with ALLN. These results show that Thr[18] and Ser[20] at the N terminus of p53, like Ser[15], Ser[33], and Ser[37], are phosphorylated in response to DNA damage. We caution, however, that in WS1, a human embryonic skin fibroblast cell line (ATTC CRL-1502), and in normal adult human fibroblasts, phosphorylation of Thr[18] appeared significantly weaker than in A549 cells even though good phosphorylation at Ser[15] was observed after exposure to ionizing radiation (data not shown). This finding suggests that the role of Thr[18] phosphorylation may be cell type-specific.

Thr[18] Phosphorylation in Vitro by Casein Kinase 1—The N terminus of mouse p53 is phosphorylated by CK1 (23, 24), a ubiquitously expressed protein kinase, the specificity of which can be directed to specific serines and threonines by phosphorylation at the −3 position (25–27). CK1 also prefers a hydrophobic residue at the +1 position. Thr[18] lies three residues downstream of Ser[15] and is followed by Phe[19]. To ascertain whether CK1 can phosphorylate Thr[18], we incubated recombinant human CK1-δ with p53(Ac-1–24) or p53(Ac-1–24) phosphorylated at Ser[15], Ser[9], and Ser[15], or at Ser[15] with Thr[18], Ser[6], Ser[9], or Thr[18] and Ser[20] changed to Ala (Fig. 4*A*). Little or no phosphorylation was observed unless Ser[15] was phosphorylated and Thr[18] was present, indicating that *in vitro* prior phosphorylation of Ser[15] specifically activates phosphorylation of Thr[18] by CK1. Phosphoamino acid analysis confirmed that Thr[18] is the major target for phosphorylation by CK1 (Fig. 4*B*). Phosphorylation of Thr[18] also was potentiated by prior phosphorylation of an N-terminal p53 fusion protein with DNA-PK (Fig. 4*C*); without phosphorylation by DNA-PK, Thr[18] was not phosphorylated by CK1, as shown by immunoblot analysis with the PAbThr(P)18 antibody.

Phosphorylation of Thr[18] in Vivo Requires Ser[15]—If CK1 is responsible for phosphorylating Thr[18] *in vivo*, then this process also should depend on the phosphorylation of Ser[15]. To address this question, we transfected H1299 lung carcinoma cells, which do not express p53 (15), with p53 expression vectors for wild-type human p53 or p53 mutated to change Ser[15] or Ser[37] to Ala (28). Cells were then either irradiated or not, and extracts were processed for immunoblot analysis with the PAbThr(P)18 antibody. Phosphorylation of Thr[18] clearly was

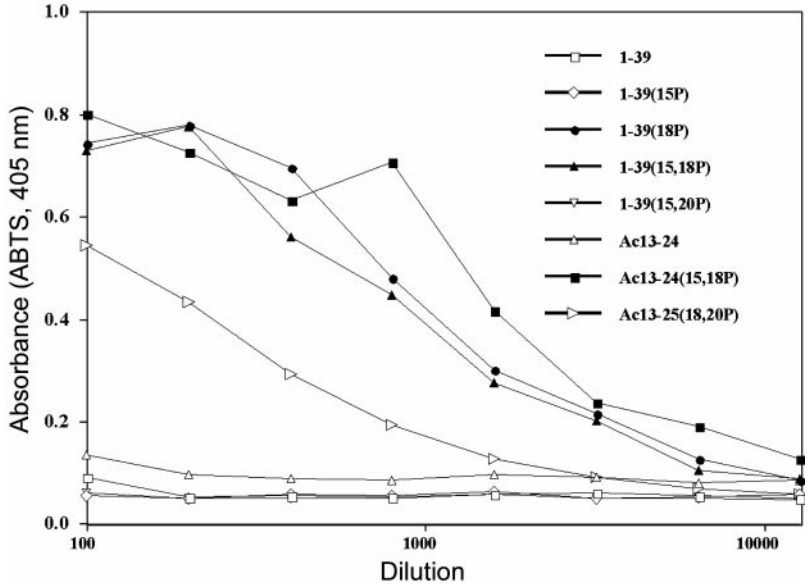

FIG. 2. **Specificity of the PAbThr(P)18 antiserum.** An affinity-purified rabbit polyclonal antibody specific for p53 phosphorylated at Thr[18] was prepared as described under "Experimental Procedures," and the antibody was evaluated by standard enzyme-linked immunosorbent assay using synthetically prepared p53 peptides. These peptides included human p53(1–39), unphosphorylated (□); p53(1–39) phosphorylated at Ser[15] (◇); p53(1–39) phosphorylated at Thr[18] (●); p53(1–39) phosphorylated at Ser[15] and Thr[18] (▲); p53(1–39) phosphorylated at Ser[15] and Ser[20] (▽); p53(13–24) unphosphorylated and acetylated at the N terminus (△); p53(Ac13–24) phosphorylated at Ser[15] and Thr[18] (■); p53(Ac13–24) phosphorylated at Thr[18] and Ser[20] (▷). This antiserum did not recognize p53(1–39) with Ser[15] changed to alanine but did recognize p53(1–39) with Ser[15] changed to alanine and Thr[18] phosphorylated (data not shown).

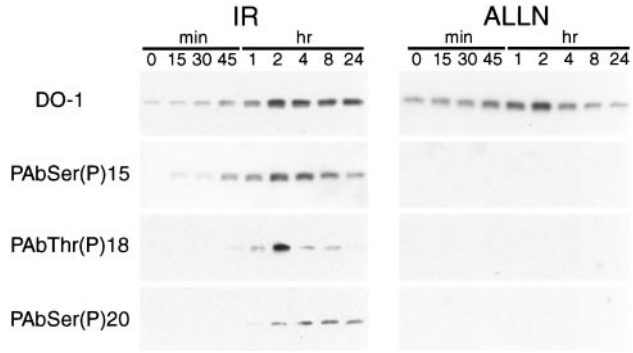

FIG. 3. **Phosphorylation of p53 at Ser[15], Thr[18], and Ser[20] in response to IR.** A549 cells were irradiated with 8 gray or treated with 20 μM ALLN, and cell extracts were prepared at the indicated times. After precipitation with PAb1801- and DO-1-agarose beads, p53 was analyzed by Western immunoblotting with the antibodies indicated at the *left*. DO-1 recognizes an epitope in the p53 transactivation domain; ALLN is an inhibitor of proteasome-mediated degradation. PAbSer(P)15, PAbThr(P)18, and PAbSer(P)20 recognize human p53 only when phosphorylated on Ser[15], Thr[18], or Ser[20], respectively, as described under "Experimental Procedures."

observed in H1299 cells transfected with wild-type p53 or with p53 that had Ser[37] changed to Ala but not in cells transfected with p53 that had Ser[15] changed to Ala (Fig. 5). In contrast, Ser[20] was phosphorylated in both mutants. No phosphorylation signal was observed from cells transfected with the vector alone. In contrast to endogenous p53 in A549 cells (Fig. 3), phosphorylation of p53 at Ser[20] and Thr[18] in transfected cells was only stimulated by and not dependent on the intentionally applied DNA damage (Fig. 5). We believe that in this case phosphorylation may be induced by the process of transfection or by DNA breaks present in the transfected DNA (29).

DISCUSSION

In vitro, Ser[15] can be phosphorylated by DNA-PK (13) and ATM (20, 30, 31), a related protein kinase that is missing or defective in people with ataxia telangiectasia (32). *In vivo* efficient phosphorylation of Ser[15] after exposing cells to IR requires a functional *ATM* gene (6), and in ATM-deficient cells, the accumulation of p53 in response to IR also is delayed (33), suggesting that phosphorylation of Ser[15] is important for stabilizing p53. An important mechanism that regulates p53 stability is its binding to Mdm2, which targets p53 for ubiquitin-mediated degradation. In contrast to other reports (5, 12, 14, 34), our data suggest that Ser[15] phosphorylation indirectly affects the association of p53 with Mdm2 rather than directly (Fig. 6). Compared with the unphosphorylated peptide, the association of p53 N-terminal peptides phosphorylated at Ser[15], Ser[37], or both, with Mdm2(17–125) was barely reduced. Importantly, however, this association was dramatically inhibited by phosphorylation at Thr[18] (Table I). The crystal structure of Mdm2(17–125) with the N terminus of p53 shows that p53 residues 18–26 form an amphipathic helix that fits into a deep hydrophobic groove in the Mdm2 structure (11). Hydrogen bonds made by the side chain hydroxyl of Thr[18] with a backbone amide and the side chain carboxyl group of Asp[21] of p53 contribute to stabilizing the helical structure of this segment. Phosphorylation of Thr[18] would eliminate these bonds and introduce a charge-charge repulsion with Asp[21] that would further destabilize the p53-Mdm2 interaction. In contrast, Ser[15] and Ser[37] lie outside the region of direct contact determined from the crystal structure. Thus, structural considerations support a role for Thr[18] phosphorylation in inhibiting the association between p53 and Mdm2. While this report was in preparation, Böttger *et al.* (35) reported that phosphorylation of Thr[18] or replacement of this threonine by amino acids other than serine substantially decreased the interaction of p53 N-terminal peptides with GST-Mdm2, in agreement with the results reported here. Moreover, Craig *et al.* (34) recently reported that monoclonal antibodies specific for phosphorylated Thr[18] detected p53 in human breast cancers with elevated p53. They also found that a peptide phosphorylated at Thr[18] inhibited p53 binding by Mdm2.

CK1-δ and CK1-ε are closely related, ubiquitously expressed mammalian homologs of budding yeast Hrr25, a nuclear Ser/Thr protein kinase that was isolated in a screen for mutants sensitive to double-stranded breaks (36–38). Yeast *HRR25* mutants are defective in the transcriptional induction of a subset of DNA damage-inducible genes that require Swi6, a transcription factor phosphorylated by Hrr25 *in vitro* (39). Murine CK1 phosphorylates several residues at the N terminus of mouse p53 (23, 24), but these residues are not completely conserved in human p53, and our attempts to phosphorylate the N terminus of unmodified human p53 with recombinant CK1-δ were unsuccessful (Fig. 4). In contrast to unmodified p53, p53(1–24) phosphorylated at Ser[15] but not Ser[9], and recombinant p53(2–47) phosphorylated at Ser[15] and Ser[37] with DNA-PK were efficiently phosphorylated by CK1 *in vitro*. These results are in

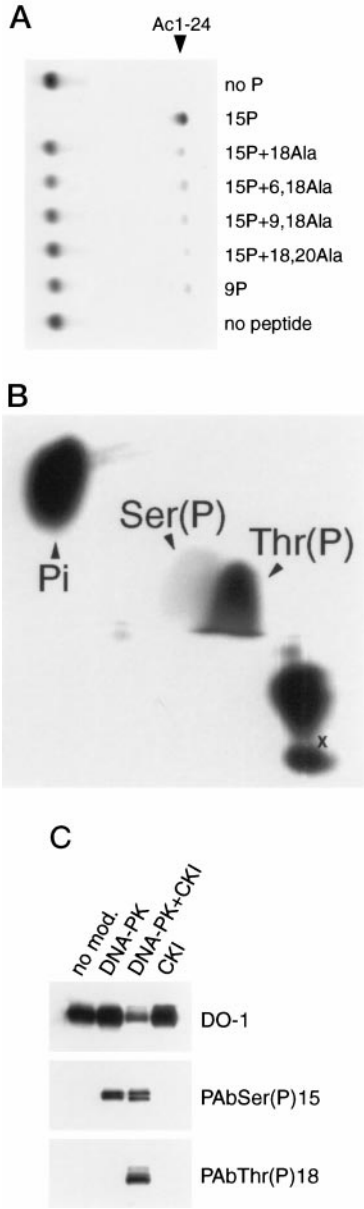

FIG. 4. **Phosphorylation of p53 N-terminal fragments by CK1.** *A*, recombinant CK1-δ and [γ-^{32}P]ATP were incubated with p53(Ac-1–24) or the indicated related peptide; the reaction products were separated by thin layer chromatography, and the phosphorylated products were detected by autoradiography. The position of the peptides are indicated (Ac-1–24). *B*, two-dimensional phosphoamino acid analysis of the p53(Ac-1–24(15P) peptide after incubation with CK1-δ. The positions of inorganic phosphate (*Pi*), phosphoserine (*Ser(P)*), phosphothreonine (*Thr(P)*), and the origin (×) are shown. *C*, a human p53(2–46)-human Oct-1 Pou-domain fusion protein was incubated with DNA-PK, CK1, or both, and the products were analyzed by immunoblotting with DO-1 or phosphorylation-specific antibodies as indicated.

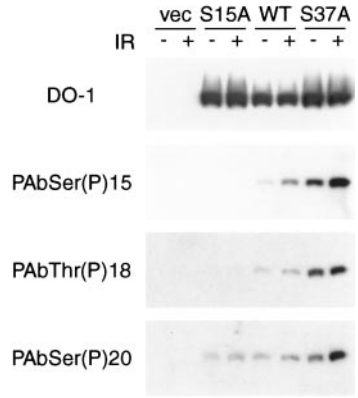

FIG. 5. **Phosphorylation of p53 Thr[18] requires Ser[15].** H1299 cells were transfected with 10 μg/plate of vector (*vec*), pC53-SN3, which expresses wild-type human p53 (43) or p53-S15A or p53-S37A (28). One day after transfection one set of cultures were exposed to 8 gray of IR; both sets were harvested for Western immunoblot analysis 2 h later. The antibodies used to detect p53 are indicated at the *left*.

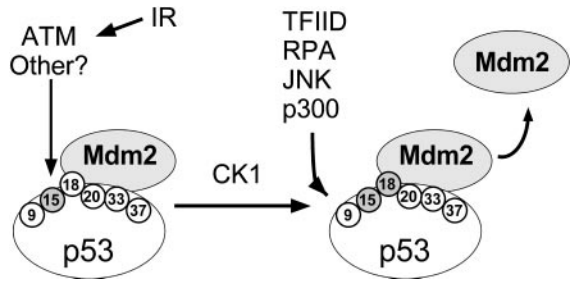

FIG. 6. **Model for inhibition of Mdm2 binding to p53 by Ser[15]-mediated phosphorylation of Thr[18] by CK1.** Human p53 becomes rapidly phosphorylated after cells are exposed to ionizing radiation (Fig. 3), possibly by ATM (20, 30). Phosphorylation of Ser[15] allows CK1 or a CK1-like enzyme to phosphorylate Thr[18]. Phosphorylation of Thr[18] inhibits binding by Mdm2, thus preventing Mdm2-mediated degradation. DNA damage-induced phosphorylation of Ser[15], Thr[18], or of other residues also may potentiate or inhibit interaction of p53 with other proteins that bind to its N terminus including RPA, TFIIH, JNK, and p300.

keeping with the known ability of phosphoserine at the −3 position to potentiate phosphorylation by CK1 (25). Phosphorylation of Ser[15] is an early event in response to IR (5, 6) (Fig. 3), and CK1 isoforms are ubiquitously expressed; thus, CK1 is a likely candidate for phosphorylating p53 Thr[18] *in vivo*. Consistent with this possibility, we found that phosphorylation of Thr[18] in response to DNA damage required the presence of Ser[15]. Curiously, this experiment also revealed that mutation of Ser[37] to alanine enhanced the level of phosphorylation at Thr[18], Ser[15], and Ser[20] (Fig. 5). The reason for this enhancement is not known but presumably reflects changes in substrate recognition for the kinases or phosphatases that control these phosphorylations and that are either dependent on Ser[37] itself or modulated by its phosphorylation. This and other recent observations indicate that there is a complex interplay between different N-terminal phosphorylation sites. For example, we recently reported that changing Ser[33] to alanine unexpectedly decreased phosphorylation levels of Ser[15] and Ser[37] (29).

Phosphorylation of Thr[18] certainly is not the only mechanism for stabilizing p53. Phosphorylation of Thr[18] after exposure to IR is transient, peaking at about 2 h (Fig. 3), and this time course is not completely coincident with p53 accumulation. Recently, Ashcroft *et al.* (40) and Blattner *et al.* (41) reported that p53 mutated to change several phosphorylation sites including Thr[18] to alanine is still stabilized after exposure to DNA damage-inducing agents. We therefore postulate that DNA damage-induced modifications to Mdm2 (42) or other components of the targeting system may contribute to stabilizing p53. Furthermore, transfection of HCT-116 p53$^{-/-}$ cells with increasing amounts of Mdm2 expressing plasmid did not differentially affect the stability of wild-type p53 compared with p53-S15A or p53-T18A (data not shown). Thus, under these conditions, phosphorylation of Thr[18] is not the dominant regulator of p53 stability. Given the central role of p53 in integrating stress-related signals and the apparent multiple mechanisms of activating p53 sequence-specific DNA binding (2, 3), the existence of redundant mechanisms for stabilizing p53 would not be surprising. In other cell types or under other

circumstances, phosphorylation of Thr[18] could play a more important role. Phosphorylation of Thr[18] and other sites at the N terminus of p53 also may regulate the interaction of p53 with other cellular proteins, such as CBP/p300, TFIIH, the TAFs, RPA, and JNK (Fig. 6), and in many cells, the regulation of such interactions may be more important. Investigations of these possibilities are in progress.

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