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Human p53 Is Phosphorylated on Serines 6 and 9 in Response to DNA Damage-inducing Agents*

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To characterize the sites in human p53 that become phosphorylated in response to DNA damage, we have developed polyclonal antibodies that recognize p53 only when it is phosphorylated at specific sites. Several attempts to generate an antibody to p53 phosphorylated at Ser^6 using a phosphoserine-containing peptide as an immunogen were unsuccessful; however, phosphorylation-specific antibodies were produced by using the phosphoserine mimetic, L-2-amino-4-phosphono-4,4-difluorobutanoic acid (F_2Pab), in place of phosphoserine. Fmoc-F_2Pab was prepared by an improved synthesis and chemically incorporated using solid phase peptide synthesis. Affinity-purified antibodies elicited by immunizing rabbits with an F_2Pab peptide coupled to keyhole limpet hemocyanin recognized a p53(1–39) peptide phosphorylated only at Ser^6 but not the unphosphorylated peptide or the same peptide phosphorylated at Ser^9, Ser^{15}, Ser^{20}, Ser^{33}, or Ser^{37}. Untreated A549 cells exhibited a background of constitutive phosphorylation at Ser^6 that increased approximately 10-fold upon exposure to either ionizing radiation or UV light. Similar results were obtained for Ser^9 using antibodies raised against a conventional phosphopeptide. Ser^9 was phosphorylated by casein kinase 1 *in vitro* in a phosphoserine 6-dependent manner. Our data identify two additional DNA damage-induced phosphorylations in human p53 and show that F_2Pab-derivatized peptides can be used to develop phosphorylation site-specific polyclonal antibodies.

The p53 tumor suppressor protein regulates cell cycle progression and cell survival in response to DNA damage and certain other cellular stresses by arresting cell cycle progression or by inducing apoptosis (1, 2). p53 normally is a short-lived protein that is maintained at low levels in unstressed cells. After cells are exposed to DNA damage-inducing agents, the p53 protein is transiently stabilized and accumulates in the nucleus where it also is activated as a transcription factor. 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 posttranslational modifications (3, 4). p53 is phosphorylated at several sites in its N-terminal transactivation domain as well as at several sites in the C-terminal tetramerization/regulatory domain, and recent studies have shown that serines 15, 20, 33, and 37 become phosphorylated after cells are exposed either to ionizing radiation (IR)[1] or to UV light, and threonine 18 becomes phosphorylated in response to ionizing radiation (5–12).

The serines at positions 4, 6, and 9 with respect to the N terminus of murine p53 are reported to be phosphorylated *in vivo* and *in vitro* by casein kinase 1 (CK1) or a CK1-like enzyme (13). CK1-δ is a homolog of the budding yeast kinase Hrr25, a nuclear Ser/Thr protein kinase that was isolated in a screen for mutants sensitive to double-stranded breaks (14–16). In human p53, serine 4 is replaced by proline, but the serines at positions 6 and 9 are conserved in most species for which sequence information is available (17). To determine whether these residues in human p53 become phosphorylated in response to DNA damage, we attempted to produce antibodies that recognize human p53 only when it is phosphorylated at these residues. Although hyperimmunization of a rabbit with a p53 peptide phosphorylated at the Ser^9 position yielded antibodies that recognized p53 peptides phosphorylated at Ser^9, repeated attempts to produce antibodies that reacted with phosphorylated Ser^6 by immunizing with a similar phosphopeptide failed. One possible reason for this failure is that the immunizing peptide becomes rapidly dephosphoryated. To circumvent dephosphorylation of the immunogen, we chemically incorporated a stable serine phosphate mimetic, F_2Pab, in place of serine phosphate in the p53 peptide used for immunizations (Fig. 1). In contrast to the natural phosphopeptide, immunization with the F_2Pab-mimetic peptide produced antisera that reacted strongly and specifically with a p53 peptide phosphorylated at Ser^6. Using the resulting affinity purified antibodies, we report here that human p53 becomes rapidly and strongly phosphorylated at Ser^6 as well as at Ser^9 in response to exposing cells to either ionizing radiation or UV light.

EXPERIMENTAL PROCEDURES

Preparation of Fmoc-F_2Pab—The intermediate, *t*-butoxycarbonyl-L-F_2Pab$(OEt)_2$-OH, was prepared in 24% yield by our improved procedure in five steps, based on the methods of Berkowitz *et al.* (18) and Otaka *et*

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[1] The abbreviations used are: IR, ionizing radiation; ALLN, *N*-acetyl-Leu-Leu-Nle-CHO; CK1, casein kinase 1; F_2Pab, L-2-amino-4-phosphono-4,4-difluorobutanoic acid; Fmoc, 9-fluorenylmethoxycarbonyl; KLH, keyhole limpet hemocyanin; ELISA, enzyme-linked immunosorbent assays.

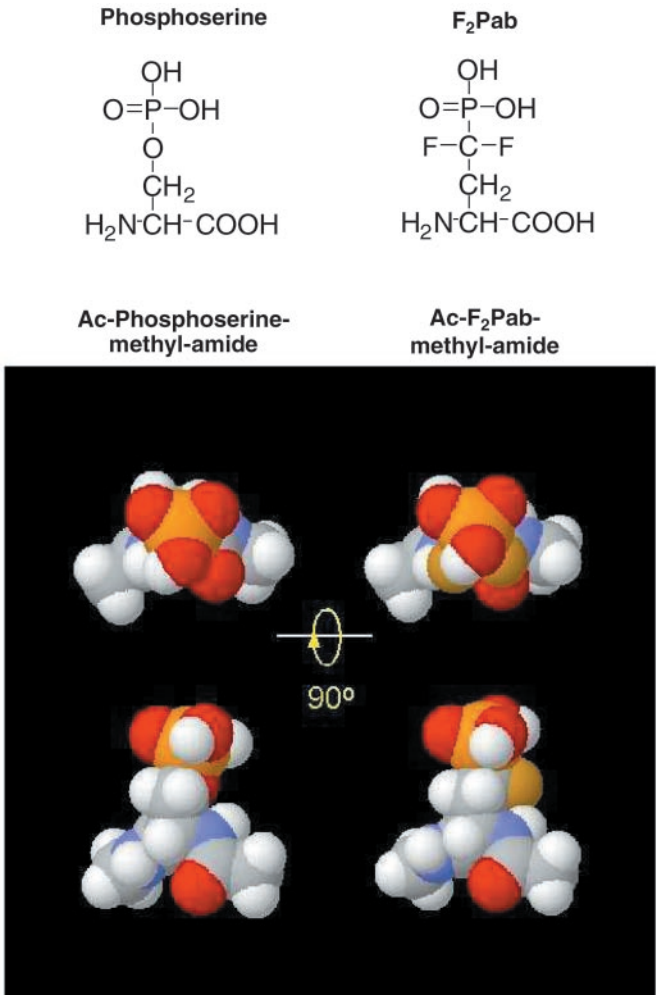

FIG. 1. **A comparison of phosphoserine and its mimetic F_2Pab.** Shown are chemical diagrams of phosphoserine and F_2Pab and two views of space-filling (Corey Pauling Koltum) models of the acetylated and methylamidated derivatives after energy minimization. Acetylation of the amino groups, methylamidation, and protonation of the phosphates simplified energy minimization calculations and approximated the local peptide environment. Energy minimization was accomplished with the Discover in Insight II program (Molecular Simulations Inc., San Diego, CA).

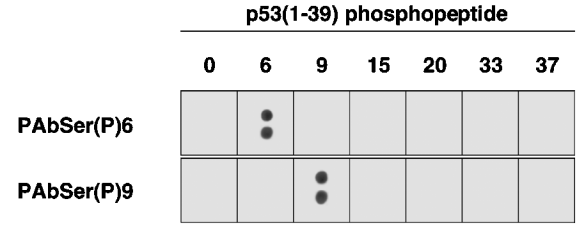

FIG. 2. **Specificity of affinity-purified PAbSer(P)6 and PAbSer(P)9 antibodies.** Affinity-purified rabbit polyclonal antibodies specific for p53 phosphorylated at Ser[6] and Ser[9] were prepared as described under "Experimental Procedures," and the antibodies were evaluated for specificity with a dot blot assay using synthetically prepared p53(1–39) and analogs phosphorylated at the sites indicated.

al. (19). Treatment of this intermediate with 1 M trimethylsilyl triflate/2 M dimethylsulfide/trifluoroacetic acid provided L- F_2Pab-OH, which was reacted with Fmoc-OSu to obtain Fmoc-L-F_2Pab-OH in 75% yield.

Peptide Synthesis and Purification—Peptides were synthesized by the solid phase method with Fmoc chemistry using an Applied Biosystems 430A peptide synthesizer (Foster City, CA). Phosphoserine was incorporated as Fmoc-Ser[PO(OBzl)OH] (Novabiochem, San Diego, CA), and L-2-amino-4-phosphono-4,4-difluorobutanoic acid (L-F_2Pab) was incorporated as Fmoc-F_2Pab-OH. The peptide was cleaved from the resin, and side chain protecting groups were removed with regent K (trifluoroacetic acid/phenol/thioanisole/H_2O/ethandithiol 82.5:5:5:5:2.5) for 3 h at room temperature. The peptides were purified by high pressure liquid chromatography on a Vydac C-8 column (Hesperia, CA) with 0.05% trifluoroacetic acid/water/acetonitrile. The mass of peptides was confirmed by electrospray ionization mass spectrometry on a Finnigan MAT SSQ 7000 (Finnigan MAT, San Jose, CA).

Antibody Preparation—Rabbit polyclonal antibody to p53 phosphorylated at Ser[6] was raised against the human p53 sequence Ac-1–12(6F_2Pab)Cys (*i.e.* Ac-MEEPQ(F_2Pab)DPSVEPC) conjugated through the added C-terminal cysteine to keyhole limpet hemocyanin (KLH). Sera from an immunized rabbit were affinity purified by use of the natural phosphopeptide Ac-1–12(6P)Cys (*i.e.* Ac-MEEPQS(P)DPSVEPC) coupled to Sulfolink (Pierce). The purified antibodies then were passed through a column coupled with the unphosphorylated peptide p53(Ac-1–12Cys) (*i.e.* Ac-MEEPQSDPSVEPC) to deplete antibodies that react with unphosphorylated p53. Antibody specific for p53 phosphorylated at Ser[9] were prepared as described (7). Rabbits were immunized with Ac-4–15(9P)Cys (*i.e.* Ac-PQSDPS(P)VEPPLSC) coupled to KLH. Phosphoserine 9-specific antibodies were purified with the use of the corresponding Sulfolinked phospho- and unphosphorylated peptides. The specificity of each antibody was confirmed by ELISA and immunoblot assays. Dot blots were prepared by spotting 1 μl of a 1 mg/ml solution of each peptide onto a nitrocellulose membrane. Membranes were blocked with 4% bovine serum albumin, 0.05% Tween 20 in phosphate-buffered saline at room temperature for 2 h, washed in phosphate-buffered saline with 0.05% Tween 20, and then processed as for Western blot analysis.

Cell Culture and Western Immunoblot Analysis of Phosphorylation Sites—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 (20), 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. Cells were seeded 24 h prior to treatment and were 60–70% confluent at the time of treatment. After being washed twice with phosphate-buffered saline, the cultures were exposed to UV irradiation using a Stratagene UV Stratalinker 2400 or to ionizing radiation using a Shepherd Mark I ^{137}Cs irradiator at a dose rate of 3.2 Gy/min. The proteasome inhibitor ALLN (Calbiochem) was added to cells at a final concentration of 20 μM as described (5); adriamycin was added to cultures at a final concentration of 0.2 μg/ml and remained in the cultures until they were harvested. At the times indicated (see Fig. 5), cultures were harvested and processed for immunoprecipitation essentially as described (7) except that 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 45 °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

For preparation of phosphorylation-specific antibodies for p53 Ser[6] and Ser[9], we first attempted to prepare phosphorylation site-specific antibodies that recognize phosphorylated Ser[6] or Ser[9] of human p53 by immunizing rabbits with chemically synthesized p53 phosphopeptides conjugated to KLH as described (7, 8). In the case of a rabbit immunized with p53(Ac-4–15(9P))C-KLH, purified antibodies were readily obtained from serum taken 6 weeks after the start of immunization that reacted specifically with the peptide p53(1–39) phosphorylated at Ser[9] but not with the unphosphorylated peptide nor with peptides phosphorylated at other known N-terminal phosphorylation sites (Fig. 2). In the case of Ser[6], however, no phosphorylation-specific antibody was obtained from any of four rabbits immunized with p53(Ac-1–12(6P))C-KLH, even after 9 weeks of immunizations. In contrast, antibodies specific for the unphosphorylated p53 peptide readily were detected in this period (Fig. 3*A*). To circumvent possible dephosphorylation of the immunizing peptide, we prepared a second peptide conjugate,

p53(Ac-1–12(6F_2Pab)C)-KLH, containing the phosphoserine mimetic, L-2-amino-4-phosphono-4,4-difluorobutanoic acid (F_2Pab) (19), in place of phosphoserine at residue 6. Immunization with this peptide yielded antibodies that recognized both an unphosphorylated p53 N-terminal peptide and an N-terminal peptide phosphorylated at Ser[6]. Indeed, the titer of antibodies in each of the initial four bleedings was higher for the phosphorylated peptide compared with the unphosphorylated peptide (Fig. 3B). Furthermore, antibodies affinity purified from serum obtained 8 weeks after the initial immunization were highly reactive and specific for p53(1–39)(6P) (Fig. 2).

To further characterize both phospho-specific antibodies, we performed quantitative ELISA assays using the purified antibodies and selected unphosphorylated and phosphorylated peptides (Fig. 4). As expected, the purified PAbSer(P)9 reacted with p53(Ac-1–12) phosphorylated at Ser[9] but not with the unphosphorylated peptide (Fig. 4, *right panel*). These antibodies also reacted well with p53(Ac-1–12) phosphorylated at both Ser[6] and Ser[9], indicating that phosphorylation at Ser[6] will not significantly affect the ability of the purified PAbSer(P)9 antibodies to recognize p53 phosphorylated at both residues. Previous studies by us and others have shown that phosphorylation at closely spaced sites can interfere with recognition by phosphorylation-specific antibodies (8). Likewise, antibodies raised against p53(Ac-1–12(6F_2Pab)) and affinity purified with the natural phospho-Ser[6] peptide (PAbSer(P)6 antibodies) recognized well p53(Ac-1–12) phosphorylated at Ser[6] or at Ser[6] and Ser[9] and did not recognize the unphosphorylated peptide (Fig. 4, *left panel*). Surprisingly, however, these antibodies hardly recognized the p53(Ac-1–12(6F_2Pab) peptide (*solid squares*). Therefore, we also purified antibodies from PAbSer(P)6 crude serum, *i.e.* from a rabbit immunized with p53(Ac-1–12(6F_2Pab)C)-KLH, on Sulfolinked p53(Ac-1–12(6F_2Pab)C) instead of the natural phosphoserine-containing peptide (Fig. 4, *middle panel*). These antibodies behaved in the opposite manner; they recognized p53(Ac-1–12(6F_2Pab) well but barely recognized the natural phospho-Ser[6] peptide. These data indicate that although F_2Pab functioned as a phosphoserine mimetic to induce the production of phosphoserine-specific antibodies, there appear to be significant differences in the structure of phosphoserine and F_2Pab or of the resulting peptides such that most antibodies that recognize phosphoserine cannot also recognize peptidyl-F_2Pab.

To determine whether human p53 is phosphorylated at Ser[6] and Ser[9] after exposure to DNA damage-inducing agents, we exposed A549 cells, a human lung carcinoma cell line with wild-type p53, to 8 Gy of ionizing radiation, to 25 J/m^2 of UV-C light, or to 0.2 μg/ml of adriamycin. As a control, cells also were treated with ALLN, a proteasome inhibitor that blocks Mdm2-mediated p53 degradation and induces the accumulation of p53 to about the same extent as DNA damage-inducing agents. Cell extracts were prepared at different times after exposure, the p53 was immunoprecipitated with a p53 antibody mixture attached to agarose beads, and the resulting immunoprecipitates

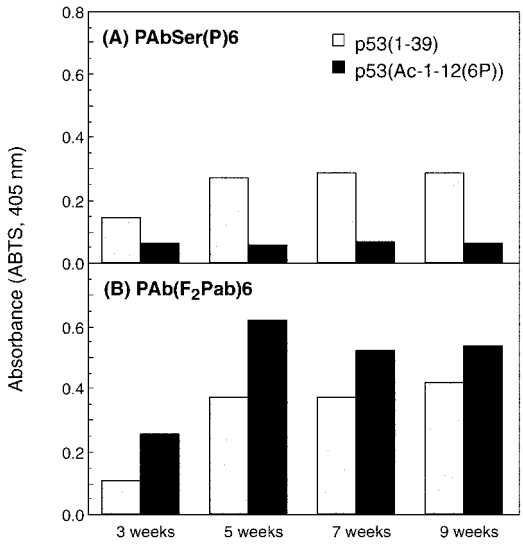

FIG. 3. **Time course of antibody development after immunization with p53(Ac-1–12(6P))C-KLH or p53(Ac-1–12(6F_2Pab))C-KLH.** Rabbits were immunized as described under "Experimental Procedures." Serum was prepared from rabbits at three (bleed 1), five (bleed 2), seven (bleed 3), and 9 weeks (bleed 4) after the initial immunization, and 5 μl of each serum was used in a standard ELISA assay with the unphosphorylated p53 N-terminal peptide p53(1–39) and with the phosphorylated peptide p53(1–12(6P)). Assays were incubated at room temperature for 60 min. The absorbance at 405 nm because of hydrolysis of the 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) substrate is proportional to the fraction of antibody in each serum that recognized the indicated substrate.

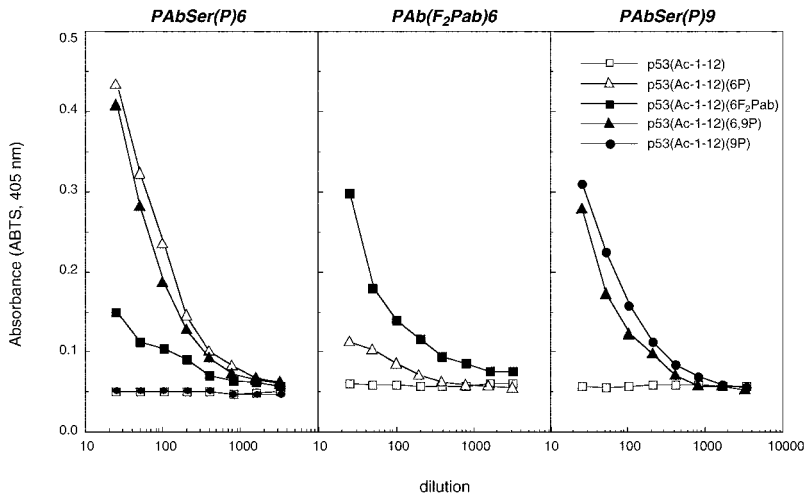

FIG. 4. **Site interactions and specificity of PAbSer(P)6, PAb(6F_2Pab)6, and PAbSer(P)9 antibodies.** Affinity-purified rabbit polyclonal antibodies specific for p53 phosphorylated at Ser[6] and Ser[9] were prepared as described under "Experimental Procedures." PAb(6F_2Pab)6 was prepared in a manner similar to how crude PAbSer(P)6 serum was prepared, except that affinity purification was with Sulfolinked Ac-1–12(6F_2Pab)Cys rather than the natural phosphopeptide. Antibodies were evaluated by standard ELISA using synthetically prepared p53 peptides. These peptides included: human p53(Ac-1–12) unphosphorylated (*open square*); p53(Ac-1–12)(6P) phosphorylated at Ser[6] (*open triangle*); p53(Ac-1–12)(6F_2Pab) with F_2Pab in place of Ser[6] (*filled square*); p53(Ac-1–12)(9P) phosphorylated at Ser[9] (*filled circle*); p53(Ac-1–12)(6, 9P) phosphorylated at both Ser[6] and Ser[9] (*filled triangle*).

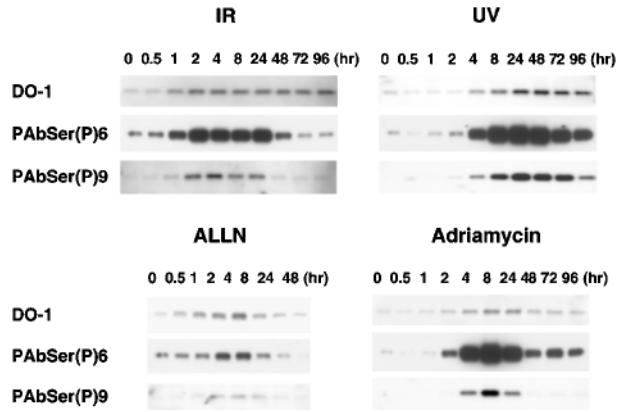

FIG. 5. **Phosphorylation of p53 at Ser[6] and Ser[9] in response to IR and UV.** A549 cells were treated with 20 μM ALLN (control), irradiated with 8 Gy, or exposed to 25 J/m^2 of UV light, and cell extracts were prepared at the indicated times. After precipitation with PAb1801- and DO-1-agarose beads, 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 and stabilizes p53 to about the same extent as DNA damage.

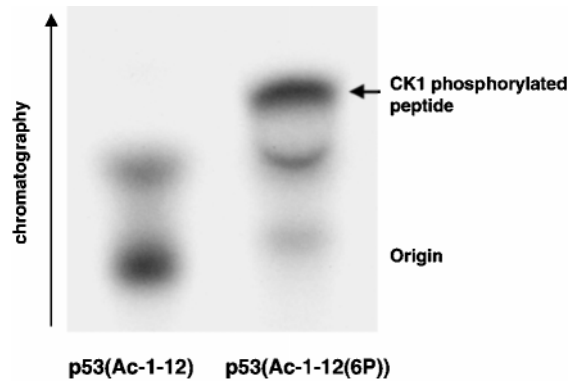

FIG. 6. **CK1-δ phosphorylates p53 Ser[9] *in vitro*.** The N-terminal human p53 peptides p53(Ac-1–12) or p53(Ac-1–12(6P)) were incubated with CK1-δ and ^{32}P-labeled ATP, and the products were separated by thin layer chromatography as described (8). The autoradiogram shows that the p53(Ac-1–12(6P)) peptide became phosphorylated (*arrow*) after incubation with recombinant CK1-δ but that the unphosphorylated was not significantly labeled (label at origin is in ATP).

were fractionated by SDS-polyacrylamide gel electrophoresis. The proteins were then electrophoretically transferred to a polyvinylidene difluoride membrane, and the membrane was probed either with phosphorylation site-specific antibody preparations or with DO-1, a monoclonal antibody that recognizes an epitope in the transactivation domain of human p53. As can been seen from Fig. 5, there was a low level of constitutive phosphorylation at both Ser[6] and Ser[9] that rose slightly after ALLN treatment approximately in parallel with the increase in p53. In sharp contrast to treatment with ALLN, there was a rapid and strong phosphorylation of Ser[6] in response to IR that was clearly observed by 1 h after irradiation and reached a plateau between 2 and 24 h before declining to near baseline values by 72 h after exposure. In other experiments (data not shown), an increase in Ser[6] phosphorylation could be observed by as little as 5 min after exposure to 8 Gy. Phosphorylation at Ser[9] in response to IR followed a similar time course to phosphorylation at Ser[6], but the apparent intensity of the damage-induced phosphorylation at Ser[9] was not as great as at Ser[6].

Ser[6] also became intensely phosphorylated in response to UV-C and to treatment with adriamycin; however, the induction of phosphorylation was slower with UV-C and adriamycin compared with induction with IR, and the response to both agents was prolonged in comparison with IR. Maximum phosphorylation of Ser[6] occurred between 8 and 48 h with UV and between 4 and 24 h with adriamycin, and residual elevated phosphorylation was still seen 96 h after exposure was initiated. Once again, the time course of induced phosphorylation at Ser[9] paralleled that at Ser[6], but phosphorylation at Ser[9] appeared to be less intense. Thus, the phosphorylation response to adriamycin was intermediate between the responses to IR and UV-C.

The similarity of the time course of phosphorylation at Ser[6] and Ser[9] suggested that phosphorylation of Ser[9] might be coupled to phosphorylation of Ser[6]. Recently, we showed that Thr[18] of human p53 is phosphorylated by a casein kinase 1-like enzyme in response to DNA damage (8). The specificity of CK1, a ubiquitously expressed protein kinase, is directed to specific serines and threonines by phosphorylation at the −3 position (21–23). Ser[9] bears a relationship to Ser[6] that is similar to the relationship between Thr[18] and Ser[15]; therefore, we asked whether CK1 also could phosphorylate Ser[9]. Fig. 6 shows that p53(Ac-1–12), as expected, was not phosphorylated by recombinant CK1-δ. However, the peptide p53(Ac-1–12)(6P), was readily phosphorylated by CK1-δ.

DISCUSSION

The response of mammalian cells to DNA damage is complex (reviewed in Refs. 24–26). Cell cycle progression is controlled by several checkpoints that are activated by DNA damage, by other stresses, and by mechanisms that ensure the orderly progression of cell cycle events. One of the most important mammalian cell cycle checkpoint proteins is the tumor suppressor p53. The *p53* gene is inactivated in the majority of human cancers (27). Studies over the last 20 years have shown that human p53 is a 393-amino acid, nuclear phosphoprotein that functions in part as an activator of transcription (reviewed in Refs. 1 and 2). In normal, undamaged cells p53 is rapidly degraded. Treating cells with a variety of DNA damage-inducing agents induces a transient accumulation of p53 protein and activates it as a transcription factor. Human p53 has been shown to be phosphorylated at several N-terminal and C-terminal sites that *in vitro* affect site-specific DNA binding and interactions with other cellular and viral proteins (3, 4).

Here we demonstrate that p53 becomes phosphorylated at two previously undescribed sites, Ser[6] and Ser[9], in response to exposing cells to ionizing radiation or adriamycin, both of which produce DNA strand breaks, and after exposure to UV light, which produces bulky lesions in DNA. Although detection of phosphorylations by Western immunoblot analysis with phosphorylation-specific antibodies is a qualitative technique, Ser[6] appears to be one of the most strongly phosphorylated sites in response to DNA damage, and this site, together with Ser[15], is one of the earliest DNA damage-induced posttranslational modifications to p53 that has been detected. Ser[9] appears to be less strongly phosphorylated, but the time course of its phosphorylation closely paralleled that of Ser[6], suggesting that phosphorylation of the two sites might be linked (see below). Studies with phosphorylated peptides suggest that phosphorylation at Ser[9] should not interfere with the detection of phosphorylation at Ser[6] and *vice versa* (Fig. 4). Serines 6 and 9 are conserved in most (*e.g.* human, monkey, mouse, rat, and hamster) but not in all (*e.g.* cow, sheep, and cat) mammalian species.

Murine p53 is phosphorylated at residues homologous to Ser[6] and Ser[9], as well as at Ser[4], which is not present in human p53, by a DNA damage-induced or activated protein kinase that resembles CK1 (4, 13). *In vitro*, recombinant CK1-δ does not phosphorylate (8), or at best phosphorylates poorly, the N

terminus of unmodified human p53 or unmodified N-terminal p53 peptides, but we recently showed that CK1-δ will phosphorylate human p53 at Thr[18] *in vitro* if it is targeted to this site by prior phosphorylation of Ser15. Ser[9], like Thr[18], is situated 3 residues distal to a site that becomes strongly phosphorylated in response to DNA damage, and Ser[9] was phosphorylated *in vitro* by CK1-δ in a Ser[6] phosphorylation-dependent manner (Fig. 6). We speculated that the phosphorylation of Ser[9] and Thr[18] by CK1-δ or a CK1-δ-like enzyme in a cascade-like manner that depends on the damage-dependent phosphorylation of the upstream −3 residue may serve as a mechanism for amplifying the biochemical effect of the initial phosphorylation. In contrast to the reported situation with murine p53, however, Ser[6] of human p53 in N-terminal peptides was not phosphorylated by CK1-δ *in vitro*. The rapid and strong phosphorylation of this site *in vivo* suggests that it is recognized by an as yet unknown DNA damage-activated protein kinase.

A significant problem in the study of p53 and its response to DNA damage has been that such studies have required labeling cells with large amounts of radioactive precursors, which themselves cause DNA damage and modification of p53 (28, 29). Antisera that specifically recognize peptidyl-phosphotyrosine have been available for several years, but antisera that specifically recognize peptidyl phosphoserine or phosphothreonine became available only recently (30). It was immediately apparent that if antibodies could be produced that recognize specific phosphorylation sites in p53, then phosphorylation at these sites could be monitored without the complication of exposing cell to high levels of DNA damaging radiation. However, producing antisera that recognized specific sites in proteins only when phosphorylated is not a trivial task, and one potentially limiting factor is dephosphorylation of the immunizing peptide by cellular phosphatases after injection of the phosphopeptide conjugates.

To circumvent the dephosphorylation problem, we explored the use of a fluorine analog of phosphoserine, F_2Pab, which can be chemically incorporated into peptides as the Fmoc-derivative at the desired site (19, 31). The F_2Pab-phosphoserine mimetic is resistant to hydrolysis by the phosphatases in cells that remove phosphates from proteins and peptides, and, in contrast to methylene derivatives, the second ionization constant of the phosphonate (pK_a2) is close to that of phosphate (32). Thus, F_2Pab-derivatized peptides appear to be stable, effective mimics of protein phosphorylation sites. Recently we showed that antibody raised to the peptide p53(13–19(15F_2Pab))A13–19(15F_2Pab))AC coupled to KLH, *i.e.* to PL(F_2Pab)QETFAPL(F_2Pab)QETFAC-KLH, was capable of specifically recognizing p53 phosphorylated at Ser[15] (8). However, we and others had previously shown that site-specific antibodies could be raised to this site using conventional phosphopeptides. In the case of Ser[6], multiple attempts to produce a phosphopeptide-specific antibody were unsuccessful even though antibodies that recognized the N-terminal segment of p53 were produced. In contrast, with the substitution of F_2Pab for the natural phosphoserine, antibodies that specifically recognized phosphorylated Ser[6] can be readily obtained.

A surprising finding is the observation that antibodies raised by immunizing with peptidyl-F_2Pab and purified using a natural phosphopeptide reacted only poorly with the immunizing peptidyl-F_2Pab peptide. Conversely, antibodies from the same bleeding that were purified using peptidyl-F_2Pab reacted only poorly with the homologous, natural phosphoserine peptide. This result suggests that F_2Pab may adopt two or more conformations, only one of which truly imitates phosphoserine. Antibodies are elicited that recognize both conformations, but these antibody populations cross-react with the alternate conformation(s) poorly. However, because we have not measured antibody recovery, there may be a third population of elicited antibodies that recognize both conformations but that were not recovered by our purification procedures. Thus, although further studies will be required to understand the nature of the immunogenic response to F_2Pab-containing peptides, our initial successes strongly suggest that the use of the F_2Pab as a mimetic for phosphoserine can provide a functional approach to developing phosphorylation-specific antisera that may even extend to developing monoclonal producing hybridomas.

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