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Ultraviolet radiation, but not γ radiation or etoposide-induced DNA damage, results in the phosphorylation of the murine p53 protein at serine-389

HUA LU*†, YOICHI TAYA‡, MASAKO IKEDA§, AND ARNOLD J. LEVINE*¶

*Department of Molecular Biology, Princeton University, Princeton, NJ 08544; ‡Biology Division, National Cancer Center Research Institute, Tsukiji 5-1-1, Chou-ku Tokyo, Japan; and §Ina Laboratories, MBL Co., Ltd., Oohara Terasawaoka 1063-103 Ina, Naganoi 396, Japan

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ABSTRACT **Polyclonal antibodies were produced and purified that selectively react with a p53 epitope containing the murine phosphoserine-389 or the human phosphoserine-392 residue, but not the unphosphorylated epitope. These antibodies, termed alpha-392, were employed to demonstrate that the phosphorylation of this serine-389 residue in the p53 protein occurs *in vivo* in response to ultraviolet radiation of cells containing the p53 protein. After ultraviolet radiation of cells in culture, p53 levels increase and concomitantly serine-389 is phosphorylated in these cells. By contrast, the serine-389 phosphorylation of the p53 protein was not detected by these antibodies in the increased levels of p53 protein made in response to γ radiation or the treatment of cells with etoposide. These results demonstrate an ultraviolet responsive and specific phosphorylation site at serine-389 of the mouse or serine-392 of the human p53 protein. Previous studies have demonstrated that this phosphorylation of p53 activates the protein for specific DNA binding. This study demonstrates *in vivo* a unique phosphorylation site in the p53 protein that responds to a specific type of DNA damage.**

The p53 tumor suppressor protein protects cells from undergoing tumorigenic alterations by inducing either cell growth arrest or program cell death in response to a variety of cellular stress signals (1, 2). One of the critical issues that remains to be elucidated is how cellular stress or DNA damage is communicated to the p53 protein so that it becomes activated and functional. It has been suggested that protein modification, such as phosphorylation of the p53 protein, may play a role in this pathway. For example, phosphorylation of the murine p53 protein at serine-389 (out of 390 aa), or its homolog serine-392 of the human p53 protein (out of 393 aa), has been shown to enhance p53 sequence-specific DNA binding *in vitro* (3), which then could activate the p53 protein for transcription. Indeed, Lozano and her colleagues (4) recently have shown that phosphorylation of serine-389/392 is important for p53-mediated transcriptional activation *in vivo*. However, previous experimental attempts to demonstrate changes in the state of phosphorylation of the p53 protein in cells treated with various DNA-damaging agents failed to document clear posttranslational alterations in the p53 protein. One possible reason for this is that exposing cells to high levels of inorganic $^{32}PO_4$ itself causes DNA damage, and so the control experiment with no other source of DNA damage (UV or γ radiation) is not a proper baseline control for comparison with cells treated with UV or γ radiation. To avoid this problem, polyclonal antibodies were developed to a specific epitope in the p53 protein (residues 386–393 in the human protein) where serine-392 was phosphorylated as described previously (5) and in the *Methods* section. These antibodies, termed α-392, reacted with both the murine (serine-389) and human phosphorylated p53 protein, but not the unphosphorylated protein. The antibodies were employed to demonstrate that p53 became phosphorylated at serine-389 (murine) after UV irradiation of cells in culture, but this site was not phosphorylated when other DNA-damaging agents, such as etoposide or γ radiation, were used. The phosphorylation of p53 selectively after UV irradiation may well be part of the signal from the damaged DNA response to p53 resulting in the activation of p53 function and an arrest in cell cycle progression (6).

METHODS

Cells and Production of Antibodies. Murine testicular carcinoma F9 cells were maintained in DMEM (GIBCO/BRL) supplemented with 10% fetal bovine serum. The α-392 antibodies were developed as described previously (5). A p53 peptide, derived from p53 amino acid residues 386 (K) to 393 (D), [CKTEGPDS(PO_3)D], was chemically synthesized by a previously described method (5) and used as the antigen. Rabbits were immunized with these phosphopeptides after conjugation with keyhole limpet hemocyanin through the cysteine residues. Polyclonal antibodies were affinity-purified from these antisera by chromatography on Sepharose CL-4b beads coupled with the same phosphopeptide, followed by passage through the beads linked with a corresponding unphosphorylated peptide. The antibodies were screened for activity by using an ELISA test (5).

Treatment of F9 Cells with UV or γ Irradiation and Etoposide. The F9 cells were cultured to 1×10^6 cell density in 150-mm dishes and subject to 20 J/m^2 of ultraviolet light as described (6) or 7 Gy of γ irradiation or 10 μM etoposide. The damaged cells were harvested at 0, 0.5, 1, 3, 7, and 24 hr after these treatments. The whole-cell extracts were prepared from the cell pellets as described previously (7), using lysis buffer containing 40 mM Tris·HCl (pH 7.9), 5 mM EDTA, 0.5% Nonidet P-40, 150 mM KCl, 2 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride, and pellets were stored at −80°C.

Purification of p53 and Glutathione *S*-Transferase (GST)-p53. The purification of human wild-type p53 from baculovirus-infected SF9 cells and the GST-p53 mutant fusion proteins containing the residues 1–82 or 320–393 of human wild-type p53 from *Escherichia coli* cells was carried out as described previously (7).

Abbreviations: ECL, enhanced chemiluminescence; GST, glutathione *S*-transferase.

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†Present address: Department of Biochemistry and Molecular Biology, Oregon Health Science University, 3181 SW Sam Jackson Park Rd., Portland, OR 97201.
¶To whom reprint requests should be addressed at: Princeton University, Department of Molecular Biology, Lewis Thomas Laboratory, Princeton, NJ 08540. e-mail: alevine@molbio.princeton.edu.

Western Blot Analysis. Western blot analysis was carried out as described previously (7). Sixty nanograms of the purified p53 and 120 ng of the GST-p53 mutant fusion protein were loaded directly onto the wells of an SDS/10% polyacrylamide gel and, after running, transferred onto a nitrocellulose membrane. The membrane was then immunoblotted with α-392 antibodies or PAb-421 antibodies directed against p53. The proteins were detected by enhanced chemiluminescence (ECL, Amersham).

Immunoprecipitation Followed by Western Blot. Immunoprecipitation was carried out as described previously (8). Cell extract protein (500 μg) from different time points was incubated with 35 μl of Sepharose CL-4B protein A beads (50% slurry) and 200 μl of the PAb 246 supernatant (approximately 2 μg purified antibodies) for 4 hr before being washed intensively as described previously (6, 8). The precipitated proteins were subject to SDS/PAGE followed by a Western blot analysis. The membranes were immunoblotted with the α -392 antibodies or PAb-421 antibodies.

RESULTS

Preparation and Specificity of Antibodies. Polyclonal rabbit antibodies were produced to p53 phosphorylated peptides as described in the *Methods* and elsewhere (5), and these antibodies were affinity-purified and termed α-392. The affinity-purified polyclonal antibodies recognized the phosphorylated form of a p53 peptide (residues 386–393) but not the unphosphorylated form employing an ELISA (results not presented). Phosphoserine-392 in the human p53 protein was chosen for this study because phosphorylation at that site stimulates sequence-specific p53 DNA binding *in vitro* (3). To test the specificity of the α-392 antibody with p53 proteins, GST-53 proteins (G-320–393 and G1–82 residues of the p53 protein) were made in *E. coli*, where they are not phosphorylated. In addition, a baculovirus p53 protein was produced, which is a mixture of phosphorylated and unphosphorylated p53 at the serine-392 site. These proteins were purified by affinity chromatography. When Western blotting procedures were used, the α-392 antibody detected the baculovirus source of the p53 protein (Fig. 1, lane 1, p53) but failed to react with the *E. coli* source of the p53 protein (Fig. 1, lane 2, G320–393, and lane 3, G1–82). By contrast, the mAb PAb421 (9), which detects an unphosphorylated p53 epitope at residues 370–381, reacted in the Western blot with p53 from baculovirus and the GST-320–393 fusion protein made in *E. coli* (Fig. 1, 421 lanes 1 and 2). Thus, the α-392 antibody is specific for the human p53 epitope at 386–393 when it is phosphorylated at serine-392 (the peptide for immunization). The same antibodies also recognized the murine p53 protein phosphorylated at residue 389 (the homolog of human serine-392), but not the unphosphorylated p53 protein.

Ultraviolet Irradiation and p53 Phosphorylation. The murine embryonal carcinoma cell line, F9, contains high levels of wild-type p53 protein that is not active as a transcription factor (10). F9 cells were exposed to UV radiation (20 J/m^2) as described previously (6), and the cells were harvested for Western blot analysis at various times after UV damage (Fig. 2). The p53 protein in the cell lysate was immunoprecipitated with the PAb246 mAb, which binds to both phosphorylated and nonphosphorylated wild-type p53 protein between residues 80 and 100 (9). The p53 protein levels and phosphorylated states then were analyzed by Western blotting by using PAb421 (epitope 370–381) or α-392 (epitope 384–393 with serine-392 phosphorylated) (Fig. 2). In agreement with previous findings (6, 10) the p53 level detected with PAb421 increased after UV irradiation at 1–3 hr postradiation with large increases at 3–7 hr (Fig. 2, 421). The α-392 antibodies also detected increased levels of phosphorylation clearly starting at 1 hr postradiation and increasing at 3–7 hr. These results demonstrate that p53 levels increase in response to UV irradiation, and phosphorylation at the murine p53 serine-389 residue (human homolog serine-392) also increases after UV irradiation with similar kinetics or with kinetics that slightly precede p53-increased levels (Fig. 2).

γ Irradiation and p53 Phosphorylation. In addition to UV irradiation (6, 10), DNA damage with γ irradiation or etoposide (11) also induces higher levels of the p53 protein, but with different kinetics after the damage. To examine the phosphorylation responses of p53 to γ irradiation or etoposide treatment, F9 cells were exposed to either 7 Gy of γ radiation or 10 μM etoposide treatment. Samples were taken at various times

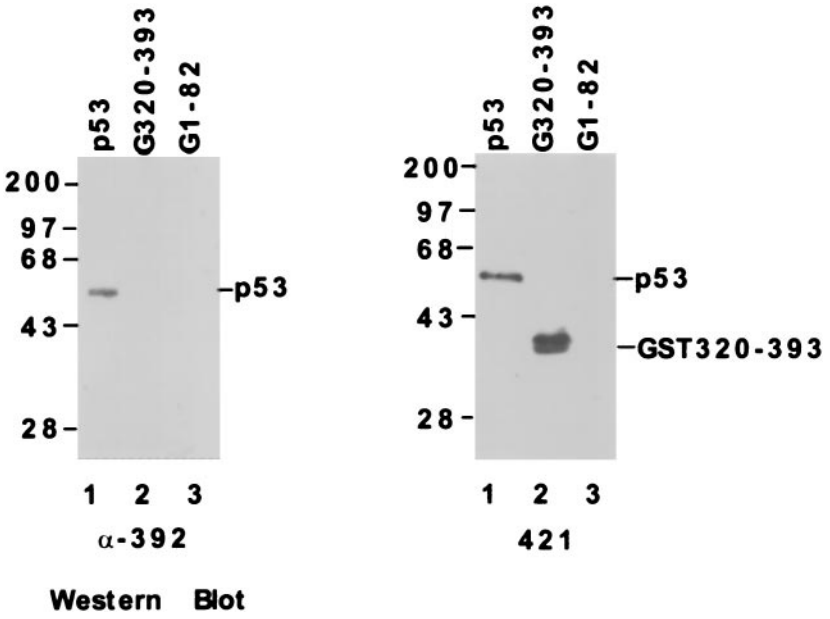

FIG. 1. The alpha-392 antibody reacts with p53 phosphorylated at serine-392 but not unphosphorylated p53. p53 phosphorylated at serine-392 was prepared in baculovirus-infected cells whereas nonphosphorylated p53 was synthesized in *E. coli* as a GST-fusion protein (G320–393, G1–82). These purified proteins were detected in a Western blot by using the alpha-392 antibody or 421 mAb. Molecular weight markers are indicated, and the positions of p53 and GST-p53 are noted.

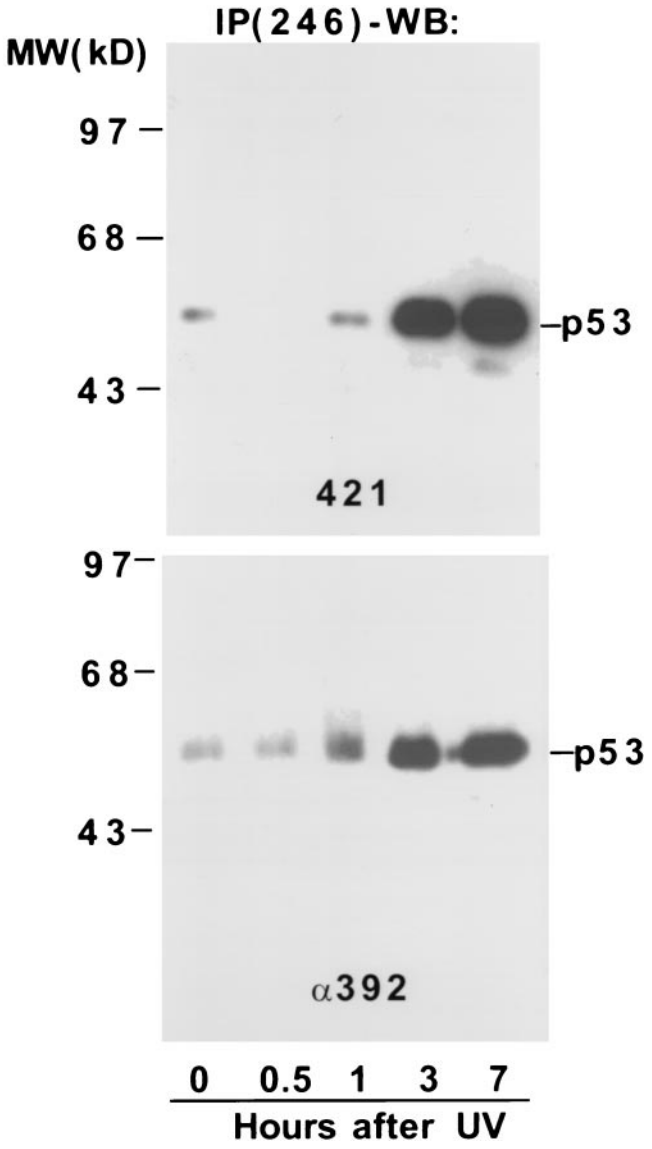

FIG. 2. Immunoprecipitation/Western blot analysis of the p53 protein from F9 cells after UV irradiation. F9 cells were irradiated with UV light (20 J/m^2) and harvested at 0, 0.5, 1, 3, and 7 hr postradiation (lanes 1–5 left to right). The p53 protein was immunoprecipitated by mAb Pab246, and the precipitate was analyzed on an SDS/polyacrylamide gel. The bands were transferred and Western blots were carried out with either Pab421 (421) or alpha-392 antibody. The position of p53 is indicated and the molecular weight markers are shown. The exposure for the ECL reagent was 10 sec for 421 and α-392.

after exposure to these agents, and the level of p53 protein and its phosphorylation at serine-389 were determined with these cell extracts (Fig. 3). As reported previously (11), p53 levels (PAb421, Fig. 3) increased at 0.5–1 hr and were maximal by 3 hr after γ irradiation (unlike UV irradiation). By contrast, the α-392 antibodies failed to detect a p53-specific band or increase in these cells after γ irradiation (Fig. 3, α-392). The bands detected by the ECL reagent in this gel were a result of the reaction with the IgG heavy chains from the PAb246 antibody and the long exposure employed (the 421 gel was exposed for 10 sec, whereas the α-392 gel was exposed for 15 min) to visualize any detectable p53 in the extract. Based on the molecular weight position (IgG, heavy chain) and the fact that a nonspecific antibody PAb419 (to simian virus 40 T-antigen) provides the same ECL result, the α-392 antibody failed to detect or find any increase in p53 phosphorylation at the serine-389 position after γ radiation (Fig. 3). The exact

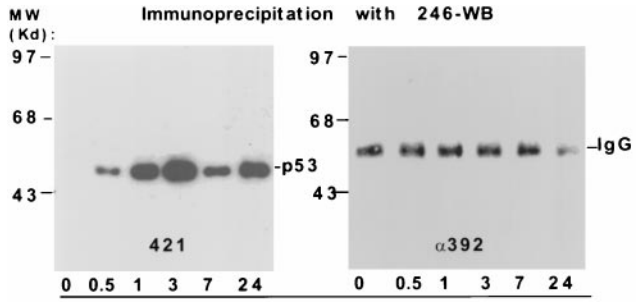

FIG. 3. Western blot analyses of p53 levels and phosphorylation patterns after γ irradiation. F9 cells were exposed to 7 Gy of γ radiation and harvested at 0, 0.5, 1, 3, 7, and 24 hr after exposure. After immunoprecipitation with Pab246, the transferred gels were probed with PAb421 (421) or α-392 antibodies. Molecular weight markers are indicated at left. The p53 bands in the 421 blot were detected with a 15-sec exposure. The IgG bands (at a higher molecule weight than p53) are present in the α-392 blot and were detected with a 15-min exposure (no bands were observed with a 15-sec exposure) used to increase the sensitivity of p53 detection. No phosphorylated form of p53 serine-389 was observed.

same result was obtained with the etoposide treatment of F9 cells in culture (results not presented). The γ irradiation and the etoposide treatment, as with the UV radiation, activated p53 as a transcription factor in these cells as monitored by the synthesis of a p53-responsive gene product, MDM-2, in these cells (results not presented; refs. 6 and 10).

DISCUSSION

Taken together, the results presented in this paper demonstrate that p53 levels increase after DNA damage with kinetics that differ between UV radiation and γ radiation or etoposide treatment. In addition to these kinetic differences (6, 11), there is a qualitative distinction: the 389 serine residue of murine p53 is specifically phosphorylated after UV irradiation but not after γ irradiation or etoposide treatment of F9 cells. The increased levels of p53 and the serine-389 phosphorylation are coincident with the transcriptional activation of p53 (10). These different responses in the phosphorylation of p53 after UV irradiation and γ irradiation correspond to the fact that the nature of DNA damage caused by these two treatments is distinct; UV produces cyclobutane pyrimidine dimers, followed by excision repair, whereas γ radiation produces single- or double-stranded breaks, followed by ligation and recombination repair. The cellular molecules that detect and repair these distinct DNA lesions are different, which could well explain why the p53 response is also different. For example, the ATM protein (ataxia telangiectasia mutant) may communicate DNA damage after γ radiation to the p53 protein (12). ATM-deficient cells lack a p53 response to γ irradiation but have a normal response to UV irradiation, giving rise to increased levels of p53 (13, 14). Similarly, the UV excision repair proteins (15–18) make up a UV-specific set of damage signaling and repair functions. Recently, the cyclin H-cdk-7 kinase –p36 complex was shown to phosphorylate p53 at four sites in the carboxyl terminus of the protein including the serine-389 site (or serine-392 of human p53) (7). The cyclin H-cdk-7 kinase is a component of TFIIH (15–18) which is both a transcriptional initiation factor and a part of the nucleotide excision repair process used after UV damage of DNA (15–18). Two of the helicases (ERCC-2 and ERCC-3) that bind to p53 (19) are subunits of TFIIH and are the proteins encoded by the xeroderma pigmentosa complementation groups B and D (16). It, therefore, appears reasonable to propose that distinct DNA damage detectors act to identify different types of DNA damage and these distinct detectors signal to different parts of the p53 protein to activate it (selectively or not) as a

transcription factor. Cyclin H-cdk-7-p36 may be used after UV damage and ATM after γ irradiation to detect single-stranded breaks in the DNA and then add phosphate groups to the p53 protein. In this way, DNA damage in the cell is communicated to the p53 protein (1, 2).

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