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Ubiquitination of p53 and p21 Is Differentially Affected by Ionizing and UV Radiation

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Levels of the tumor suppressor protein p53 are normally quite low due in part to its short half-life. p53 levels increase in cells exposed to DNA-damaging agents, such as radiation, and this increase is thought to be responsible for the radiation-induced G_1 cell cycle arrest or delay. The mechanisms by which radiation causes an increase in p53 are currently unknown. The purpose of this study was to compare the effects of gamma and UV radiation on the stability and ubiquitination of p53 in vivo. Ubiquitin-p53 conjugates could be detected in nonirradiated and gamma-irradiated cells but not in cells which were UV treated, despite the fact that both treatments resulted in the stabilization of the p53 protein. These results demonstrate that UV and gamma radiation have different effects on ubiquitinated p53 and suggest that the UV-induced stabilization of p53 results from a loss of p53 ubiquitination. Ubiquitinated forms of p21, an inhibitor of cyclin-dependent kinases, were detected in vivo, demonstrating that p21 is also a target for degradation by the ubiquitin-dependent proteolytic pathway. However, UV and gamma radiation had no effect on the stability or in vivo ubiquitination of p21, indicating that the radiation effects on p53 are specific.

Mutations in the gene encoding the tumor suppressor protein p53 are the most common genetic alterations detected in human cancer (22). A role for p53 in normal cellular proliferation has not been clearly identified. However, there is abundant evidence that wild-type p53 plays a critical role in the cellular response to DNA damage by serving as a cell cycle checkpoint determinant (reviewed in reference 6). p53 levels increase in cells exposed to ionizing radiation (IR) (29, 30, 55), and the cells undergo a cell cycle arrest or delay in the G_1 phase of the cell cycle (30, 55). This arrest or delay is thought to allow time for the cells to repair the DNA damage incurred during IR treatment before proceeding in S phase and thereby prevent the accumulation of mutations that would result from replicating a damaged genome. Cells which lack wild-type p53 function fail to arrest following radiation treatment (26–28, 30, 55), indicating an essential role for p53 in the arrest response. The p53-dependent G_1 arrest appears to be mediated by p21 (2, 3, 8), a cyclin-dependent kinase inhibitor whose gene is transcriptionally activated by p53 (11, 18, 57). p53 also plays a role in the signaling of apoptosis (programmed cell death) in certain cell types following irradiation treatment (1, 32). For example, thymocytes from p53 knockout mice were less susceptible to radiation-induced apoptosis than were thymocytes from mice expressing p53 (32). This apoptotic function may involve the ability of p53 to activate transcription of the gene encoding bax, a protein which is involved in an apoptosis signaling pathway (17). There is increasing evidence that p53 carries out a similar checkpoint function during normal cell division. For example, cells which lack wild-type p53 have high levels of genomic instability (7, 10, 12, 52), accumulate mutations at an increased rate (19), and are at an increased risk for malignant progression (9, 24). Based on these findings, it has been proposed that the normal function of p53 is to monitor the integrity of the genome and protect cells from accumulating genetic damage. p53 carries out this function by temporarily halting the cell cycle in response to aberrations in the genetic material, thereby allowing cells time to repair the DNA before proceeding with cell division.

Levels of wild-type p53 are usually quite low due to a short protein half-life. However, p53 protein is stabilized in response to various DNA-damaging agents, and its level increases. The turnover of p53 in vivo is mediated by the ubiquitin proteolysis system (35), and enzymes which can participate in the in vitro ubiquitination of p53 have been identified (48–50). Ubiquitination of a protein involves three separate enzymatic activities, designated E1, E2, and E3 (reviewed in references 5, 20, and 21). Ubiquitin is first activated through its covalent thio-ester linkage to the E1 ubiquitin-activating enzyme. The activated ubiquitin is then transfered to the E2 enzyme, also known as a ubiquitin-conjugating enzyme (UBC), again in the form of a high-energy thio-ester bond. In some cases the activated ubiquitin is transferred from the E2 enzyme to an E3 protein, also known as a ubiquitin protein ligase, which in turn transfers the activated ubiquitin directly to a substrate (50). In other cases the E2 enzyme may transfer the ubiquitin to the substrate protein, while the E3 protein aids in recognition of the substrate by the E2. Additional ubiquitin moieties are linked sequentially to each other, leading to the formation of multiubiquitin chains, and the multiubiquinated substrate is then degraded by the 26S proteasome. The E6 proteins of cancer-associated human papillomaviruses (i.e., types 16 and 18), in association with a cellular factor termed E6AP, can complex p53 in vitro (23) and promote its ubiquitination and subsequent degradation by the proteasome (48–50). Recently it has been demonstrated that E6AP, in the presence of E6, can transfer activated ubiquitin directly to p53 (50). The E6-E6AP complex functions as an E3 protein in the in vitro ubiquitination of p53. It is unknown at present if E6AP plays a role in the normal turnover of p53 in the absence of E6.

The mechanism(s) by which DNA damage causes a stabilization of p53 is unknown. However, given that wild-type p53 is degraded via the ubiquitin proteolysis pathway, one possibility is that DNA-damaging agents somehow signal a repression of the ubiquitin-mediated degradation of p53. p53 levels have been reported to increase in response to a large number of

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DNA-damaging or DNA-modifying agents in addition to IR, including UV light (36), actinomycin D (28), cisplatin (44), etoposide (25, 56), and mitomycin (25, 42), among others. The wide variety of agents capable of inducing p53 raises the question as to the nature of the inducing signal and whether various DNA-damaging regimens affect p53 levels through common mechanistic pathways. Lu and Lane reported that the kinetics and magnitude of p53 induction were grossly different in cells in response to either IR or UV (34). These differences may reflect different mechanisms by which p53 is induced following either IR or UV treatment. More recent studies have suggested that IR treatment positively affects p53 mRNA translation (41). The current study was undertaken to examine the effects of exposure to gamma rays (a form of IR) and UV light on p53 levels, stability, and in vivo ubiquitination. p53-ubiquitin conjugates were lost shortly after exposure to UV but not after exposure to gamma radiation, despite the fact that p53 was stabilized in response to both treatments. In contrast, UV or gamma radiation did not affect the stability or in vivo ubiquitination of p21, which we report is also degraded via the ubiquitin pathway. These results demonstrate that UV and gamma radiation have differential effects on ubiquitinated p53 and suggest that the UV-induced stabilization of p53 is due to a specific loss of p53 ubiquitination.

MATERIALS AND METHODS

Cell strains, tissue culture, and transfections. RKO cells were from Michael Brattain (Department of Biochemistry, Medical College of Ohio). Saos-2 cells are a human osteosarcoma cell line that does not express p53, and U2OS cells are a human osteosarcoma cell line that expresses wild-type p53. RKO cells were grown in McCoy's 5A medium containing 10% fetal bovine serum. All other cell strains were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. All radiation treatments were carried out at the Laboratory of Radiobiology, Harvard School of Public Health. IR was carried out using a ^{60}Co gamma ray source (U.S. Nuclear) at a dose rate of 13 cGy/s. The UV light exposure apparatus consisted of five UV bulbs which delivered 254-nm light at a dose of 2.08 J/m^2/s. All proteasome inhibitors used in this study were kindly provided by Proscript Inc., Boston, Mass. The inhibitor lactacystin (13), as well as the peptide aldehydes MG115 (Z-Leu-Leu-Nva-H) and MG132 (Z-Leu-Leu-Leu-H) (47), has been described elsewhere. Proteasome inhibitors in dimethyl sulfoxide were added directly to the culture media to a final concentration of 0.025 mM. Cycloheximide (Sigma) was dissolved in absolute ethanol and added directly to the culture media to a final concentration of 20 or 25 μg/ml. Transfection of U20S or Saos-2 cells was done by the calcium-phosphate transfection method when the cells were approximately 60% confluent. Ubiquitin expression plasmids pMT-107 and pMT-123 (54) were kindly provided by Dirk Bohmann. The mammalian expression plasmid encoding full-length wild-type p21 (18) contains the p21 cDNA in the *Eco*RI site of pcDNA-3 (Invitrogen). The p21 construct with the C-terminal 11 amino acids deleted was generated by PCR amplification from the full-length clone using the T7 promoter primer (5′TAA TACGACTCACTATAGGG3′) encoded by the plasmid and the δ11-C primer (5′CCGCCCGCGGCCGCTTAGGAGTGGTAGAAATATG3′). The resulting PCR fragment was cleaved at the *Bam*HI site of pCDNA-3 and a *Not*I site contained in the δ11-C primer and cloned into the corresponding sites of pcDNA-3. Two micrograms of each p21 expression plasmid was transfected alone or cotransfected with 6.5 μg of the ubiquitin expression plasmid pMT-107 or pMT-123. In each transfection, the final DNA amount was adjusted to 15 μg by the addition of herring sperm DNA.

SDS-PAGE, Western blots, and immunoprecipitations. For Western blot analysis without prior immunoprecipitation, cells were washed twice with phosphate-buffered saline, scraped into 0.5 ml of lysis buffer (50 mM Tris [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride), and incubated on ice for 15 min with occasional light vortexing. Cells were then sonicated for 10 pulses at setting 5, 50% output, with a Branson 450 sonifier. Lysates were spun at 15,000 × *g* for 15 min to remove cellular debris. One hundred micrograms of protein extract from the resulting supernatants was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P membranes (Millipore) for detection with p53 or p21 antibodies. For detection of either p53- or p21-ubiquitin protein conjugates, cell lysates were prepared in radioimmunoprecipitation (RIPA) buffer (2 mM Tris [pH 7.5], 5 mM EDTA, 150 mM NaCl, 1.0% Nonidet P-40, 1.0% deoxycholate, 0.025% SDS, 1 mM phenylmethylsulfonyl fluoride) and immunoprecipitated with either p53 antibody Ab-6 or Ab-421 or the anti-p21 polyclonal antibody 15431E (Pharmingen). The immunoprecipitates were resolved by SDS-PAGE and transferred to an Immobilon-P membrane, and the membrane was then autoclaved in water for 15 min. For p53 detection, the membrane was cut at approximately 60 kDa, and the upper portion of the membrane was examined by Western blotting with anti-p53 monoclonal antibody Ab-6, Ab-421, or Ab-1801 (Oncogene Science). For detection of p21, the blots were probed with anti-p21 monoclonal antibody 15091A (Pharmingen). The antiubiquitin monoclonal antibody M43 (16) used in these experiments was generously provided by Linda Guarino (Texas A&M University).

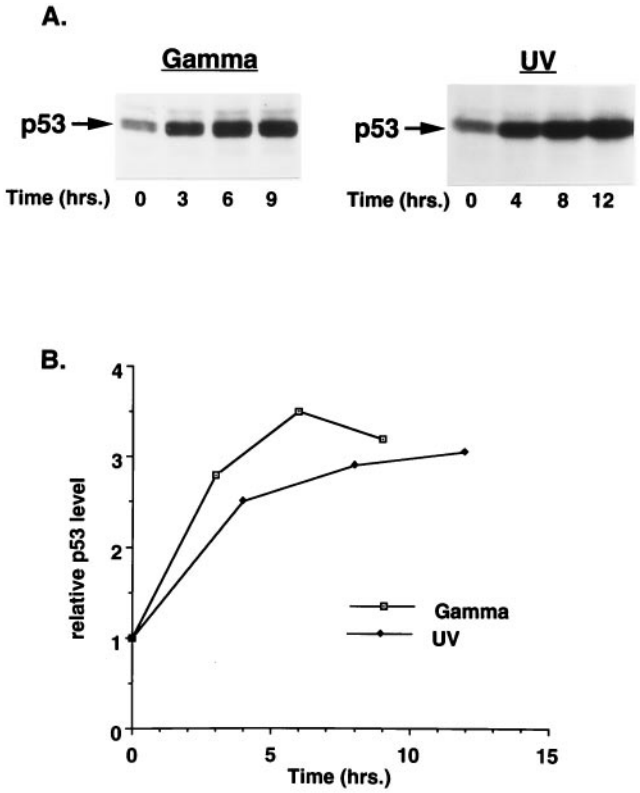

FIG. 1. (A) p53 levels increase in response to irradiation. RKO cells were gamma irradiated at a dose of 10 Gy or UV irradiated at a dose of 20 J/m^2. Cell lysates were prepared at the indicated times after irradiation and examined by Western blotting with the p53-specific antibody Ab-6. (B) p53 levels in panel A were quantitated by densitometric scanning of the autoradiograms and are plotted. The p53 level at the zero time point in each case is given a relative value of 1.0.

RESULTS

p53 levels increase following gamma or UV irradiation. Wild-type p53 levels increase in cells exposed to various DNA-damaging agents, including gamma rays (a form of IR) and UV radiation. The purpose of this study was to examine the mechanisms accounting for this increase and to characterize the effects of gamma and UV radiation on p53. The human colon cancer cell line RKO was used in these experiments because it expresses wild-type p53 (26, 27), the DNA damage response of p53 following radiation treatment in RKO cells has been well documented, and the p53-mediated growth arrest pathway following radiation is functionally intact (26–28, 30). p53 can be detected as a doublet by Western blot analysis (see Fig. 1, 4, and 5) or by metabolic labeling of RKO cells (not shown) (see Fig. 1, 2, 4, and 5), presumably representing differentially phosphorylated forms of p53. As illustrated in the Western blots depicted in Fig. 1, the steady-state levels of p53 in RKO cells increased three- to fourfold following either gamma or UV radiation, and both p53 species were susceptible to this DNA damage-induced increase.

Stabilization of p53 by gamma and UV irradiation. Metabolic labeling experiments and Northern blot analyses were done to determine the effects of UV and gamma irradiation on p53 synthesis. UV irradiation had no observable effect on p53 mRNA levels or synthesis rates, and gamma irradiation had only a slight effect on p53 synthesis (not shown). These results suggested that the increased p53 levels following UV or gamma irradiation must involve stabilization of the protein. The stability of p53 was therefore examined in RKO cells exposed to either UV or gamma radiation under conditions which induce increased steady-state levels of p53. After radiation, the cells were treated with cycloheximide to inhibit de novo p53 synthesis, and the steady-state levels of p53 were determined at the indicated times (Fig. 2). Under these conditions, the rate at which p53 steady-state levels decline reflects the half-life of the protein. The half-life of p53 in nonirradiated RKO cells was between 30 and 60 min. In contrast, the half-life of the p53 protein was extended to greater than 3.5 h in cells following exposure to either gamma or UV radiation, indicating that both UV and gamma radiation cause a stabilization of the p53 protein.

Effects of gamma and UV irradiation on p53 ubiquitination in vivo. We recently reported the detection of ubiquitin-p53 conjugates in vivo, providing direct evidence that the normal turnover of wild-type p53 can occur via the ubiquitin-proteolytic pathway (35). To determine whether the stabilization of p53 following gamma and UV radiation was due to an inhibition of the ubiquitination of p53 in vivo, p53 protein from RKO cells that were either untreated or treated with gamma or UV radiation was examined by Western blot analysis using antibodies specific to either ubiquitin or p53 (Fig. 3). The ubiquitin antibody detected a ladder of p53-specific bands from both untreated and gamma-irradiated RKO cells (Fig. 3A, left). These same bands were detected by the p53 monoclonal antibody Ab-6, which recognizes an epitope on p53 distinct from that recognized by Ab-421 used for immunoprecipitation (Fig. 3A, right), confirming the specificity for p53 and confirming that the ladder of bands recognized by both the ubiquitin and p53 antibodies are ubiquitinated p53 species. The sizes of the bands ranged from approximately 69 to 92 kDa, which corresponds to species of p53 conjugated to two to five ubiquitin molecules. A similar ladder of ubiquitinated p53 bands was also detected in both nonirradiated and gamma-irradiated U2OS cells, a human osteosarcoma cell line that also expresses wild-type p53 (14, 27) (Fig. 3C). The ubiquitinated p53 species were detectable 5 to 6 h after gamma radiation treatment, at time points when the steady-state levels of p53 were increased (Fig. 3B and 1). The ubiquitinated p53 species were not detected in RKO or U2OS cells following UV irradiation. The ubiquitinated p53 species disappeared as early as 1 h following UV irradiation (Fig. 3). These results demonstrate that gamma radiation and UV radiation have different effects on ubiquitinated forms of p53 and suggest that the UV-induced stabilization of p53 is due to a specific loss of p53 ubiquitination. As demonstrated in Fig. 4, ubiquitinated forms of p53 could still be detected at low UV doses (5 J/m^2) but were completely lost at higher UV doses (10 and 20 J/m^2). These results indicate that the UV-induced loss of p53 ubiquitination was dose dependent.

p21 is ubiquitinated in vivo. To determine the specificity of the UV-induced inhibition of p53 ubiquitination, the effect of UV radiation on another protein that is degraded by the ubiquitin system was examined. We have shown that p21, a cyclin-dependent kinase inhibitor, is stabilized in cells exposed to specific inhibitors of the proteasome, suggesting that p21 is also a target of the ubiquitin system. Two approaches were taken to detect ubiquitinated forms of p21 in vivo. The first approach involved the transient overexpression of ubiquitin in cells, followed by treatment of the cells with specific inhibitors of the proteasome. U2OS cells were used in these experiments because they express a detectable amount of p21 protein and because they can be transfected at a high efficiency. U2OS cells that were either untransfected or transfected with a ubiquitin expression plasmid were treated with a proteasome inhibitor, MG115 (47) or lactacystin (13). The p21 protein was immu-

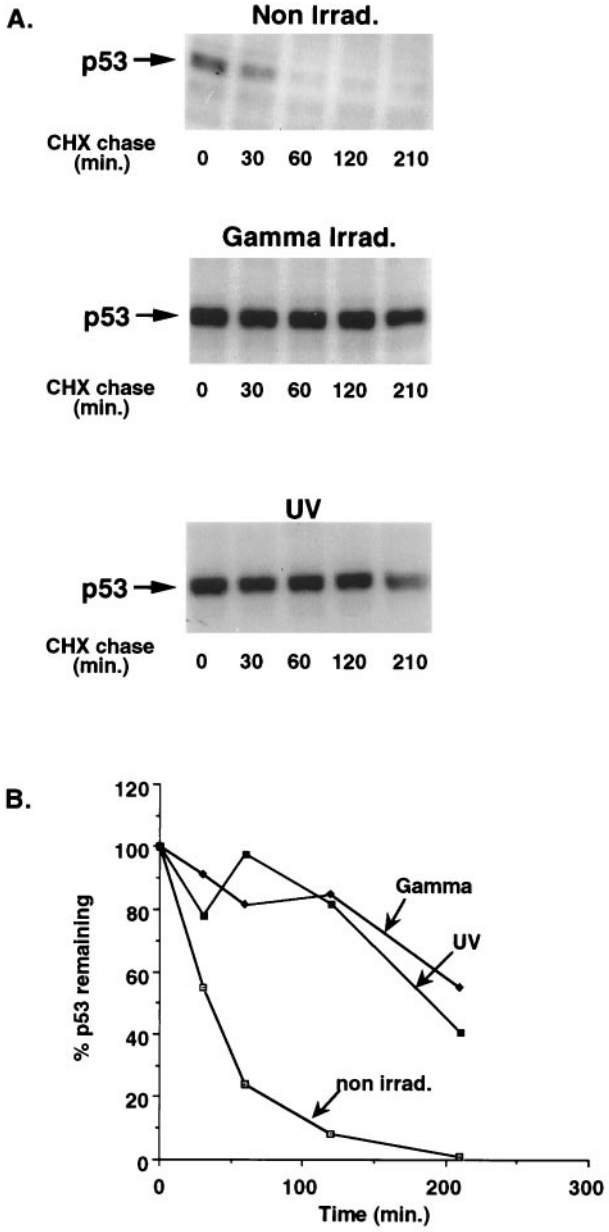

FIG. 2. (A) Gamma radiation and UV irradiation (Irrad.) stabilize p53. RKO cells were either nonirradiated or gamma irradiated (10 Gy) or UV irradiated (20 J/m^2). At 4 h after irradiation treatment, cycloheximide (CHX) was added to a final concentration of 20 μg/ml. At the indicated times after addition of CHX cell lysates were prepared and examined by Western blot analysis with the p53-specific antibody Ab-6. (B) p53 levels from panel A were quantitated by densitometry and plotted. The half-life of p53 in nonirradiated cells was between 30 and 60 min and was extended to greater than 210 min in gamma- and UV-irradiated cells.

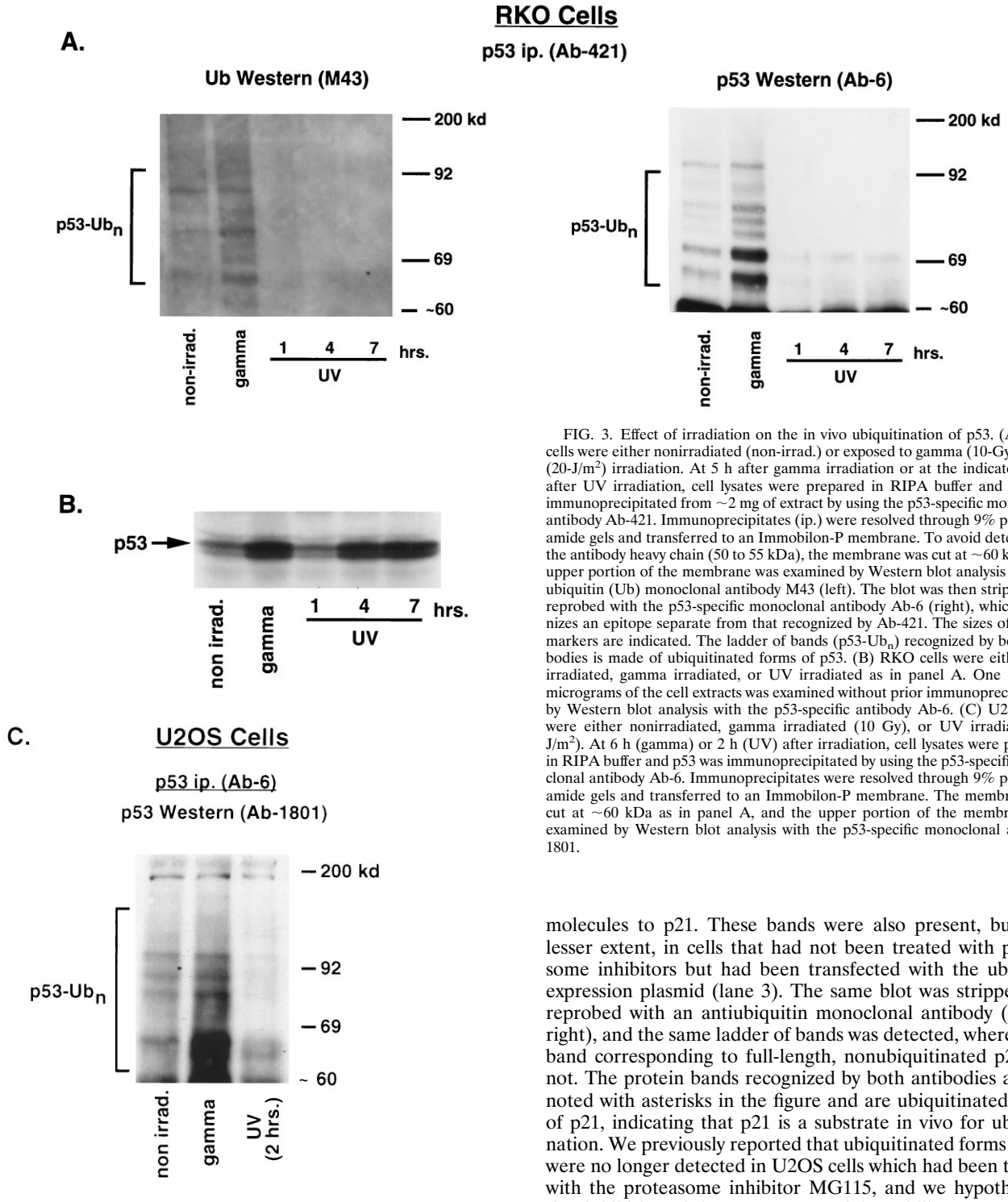

FIG. 3. Effect of irradiation on the in vivo ubiquitination of p53. (A) RKO cells were either nonirradiated (non-irrad.) or exposed to gamma (10-Gy) or UV (20-J/m^2) irradiation. At 5 h after gamma irradiation or at the indicated times after UV irradiation, cell lysates were prepared in RIPA buffer and p53 was immunoprecipitated from ~2 mg of extract by using the p53-specific monoclonal antibody Ab-421. Immunoprecipitates (ip.) were resolved through 9% polyacrylamide gels and transferred to an Immobilon-P membrane. To avoid detection of the antibody heavy chain (50 to 55 kDa), the membrane was cut at ~60 kDa. The upper portion of the membrane was examined by Western blot analysis with the ubiquitin (Ub) monoclonal antibody M43 (left). The blot was then stripped and reprobed with the p53-specific monoclonal antibody Ab-6 (right), which recognizes an epitope separate from that recognized by Ab-421. The sizes of protein markers are indicated. The ladder of bands (p53-Ub_n) recognized by both antibodies is made of ubiquitinated forms of p53. (B) RKO cells were either nonirradiated, gamma irradiated, or UV irradiated as in panel A. One hundred micrograms of the cell extracts was examined without prior immunoprecipitation by Western blot analysis with the p53-specific antibody Ab-6. (C) U2OS cells were either nonirradiated, gamma irradiated (10 Gy), or UV irradiated (20 J/m^2). At 6 h (gamma) or 2 h (UV) after irradiation, cell lysates were prepared in RIPA buffer and p53 was immunoprecipitated by using the p53-specific monoclonal antibody Ab-6. Immunoprecipitates were resolved through 9% polyacrylamide gels and transferred to an Immobilon-P membrane. The membrane was cut at ~60 kDa as in panel A, and the upper portion of the membrane was examined by Western blot analysis with the p53-specific monoclonal antibody 1801.

noprecipitated with a p21 polyclonal antibody, and the immunoprecipitates were examined by Western blot analysis with monoclonal antibodies against p21 or against ubiquitin (Fig. 5). Proteasome inhibitor treatment of U2OS cells (either transfected or untransfected with the ubiquitin expression plasmid) resulted in the appearance of a ladder of bands which were recognized by the p21 monoclonal antibody and whose sizes were consistent with the addition of between 2 and 10 ubiquitin molecules to p21. These bands were also present, but to a lesser extent, in cells that had not been treated with proteasome inhibitors but had been transfected with the ubiquitin expression plasmid (lane 3). The same blot was stripped and reprobed with an antiubiquitin monoclonal antibody (Fig. 5, right), and the same ladder of bands was detected, whereas the band corresponding to full-length, nonubiquitinated p21 was not. The protein bands recognized by both antibodies are denoted with asterisks in the figure and are ubiquitinated forms of p21, indicating that p21 is a substrate in vivo for ubiquitination. We previously reported that ubiquitinated forms of p53 were no longer detected in U2OS cells which had been treated with the proteasome inhibitor MG115, and we hypothesized that inhibition of the proteasome might stabilize an activity in U2OS cells which deubiquitinates p53 (35). The current results show that ubiquitinated forms of p21 accumulate in U2OS cells treated with the proteasome inhibitor. These results demonstrate that inhibition of the proteasome has different effects on the ubiquitinated forms of p21 and p53.

The second approach to detect ubiquitinated p21 in vivo involved the introduction of DNAs expressing both wild-type p21 and ubiquitin into cells. An approach similar to this was used previously to detect ubiquitinated c-Jun in vivo (54).

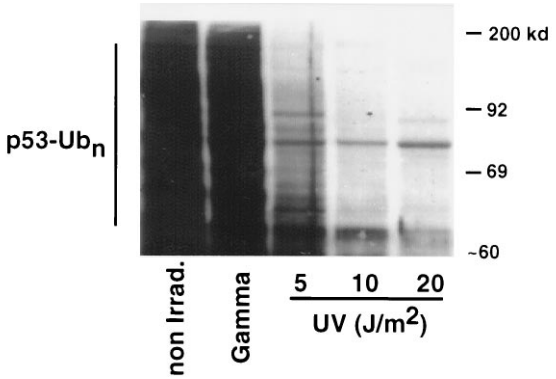

FIG. 4. Dose-dependent effect of UV irradiation on ubiquitinated p53. RKO cells were either nonirradiated (non Irrad.), gamma irradiated (10 Gy), or UV irradiated at the indicated dose. At 4 h after irradiation, cell lysates were prepared in RIPA buffer and p53 was immunoprecipitated from ~2 mg of extract by using the p53-specific monoclonal antibody Ab-6. Immunoprecipitates were resolved through 9% polyacrylamide gels and transferred to an Immobilon-P membrane. The membrane was cut at ~60 kDa as in Fig. 3, and the upper portion of the membrane was examined by Western blot analysis with the p53-specific monoclonal antibody Ab-421. The gel is overexposed in order to see the bands when the UV dose was 5 J/m^2.

Saos-2 cells were used in these experiments because they do not express detectable levels of endogenous p21, and it would therefore be possible to monitor only the ubiquitination of the transfected p21 protein. A plasmid expressing p21 was transfected alone or together with a plasmid encoding ubiquitin into Saos-2 cells. The cells were treated with a proteasome inhibitor, MG115 or MG132 (47), and p21 immunoprecipitates were examined by Western blot analysis using a monoclonal antibody to p21 (Fig. 6). A protein species which migrated slightly above 30 kDa and which was recognized by the p21 monoclonal antibody was observed in p21-transfected cells treated with the proteasome inhibitor (Fig. 6). The size of this species is consistent with addition of two ubiquitin molecules to p21. This same band was also observed in cells transfected with the p21 and ubiquitin expression DNAs but not in cells transfected with the ubiquitin expressor alone or in cells treated with the proteasome inhibitor alone. To verify that this band was derived from p21, the experiment was carried out using a p21 protein with its C-terminal 11 amino acids deleted (Fig. 6B). Coexpression of p21δ11C with the ubiquitin plasmid resulted in a smaller band migrating at a size consistent with the deletion of 11 amino acids. This confirms that this protein band is derived from p21. The fact that the intensity of this modified form of p21 increased in cells treated with the proteasome inhibitors and in cells overexpressing ubiquitin is consistent with its being a ubiquitinated form of p21. The higher-molecular-mass protein bands of ~40 to 50 kDa (marked by asterisks in Fig. 6) observed in the proteasome inhibitor-treated cells coexpressing p21 and ubiquitin most likely represent more highly ubiquitinated forms of p21.

Ubiquitination of p21 in irradiated cells. Having established that p21 can be ubiquitinated in vivo, the effects of gamma and UV irradiation on the in vivo ubiquitination of p21 were next examined. U2OS cells were irradiated and subsequently treated with the proteasome inhibitor MG132 to examine the effects of gamma and UV irradiation on the ubiquitination of p21 (Fig. 7). Ubiquitinated p21 species were clearly evident in U2OS cells treated with MG132. Low levels of ubiquitinated p21 were also detected in gamma-irradiated cells, and this level of ubiquitinated p21 was increased in gamma-irradiated cells treated with MG132. Ubiquitinated p21 species were not detected in UV-irradiated cells; however, ubiquitinated forms of p21 did accumulate in U2OS cells which were UV irradiated and subsequently treated with MG132. As mentioned earlier, ubiquitinated forms of p53 could no longer be detected in U2OS cells treated with a proteasome inhibitor (35), suggesting that proteasome inhibition stabilizes an activity which deubiquitinates p53. Not surprisingly, ubiquitin-p53 conjugates were no longer detected in U2OS cells treated singly with MG132 or UV radiation or in cells UV irradiated and subse-

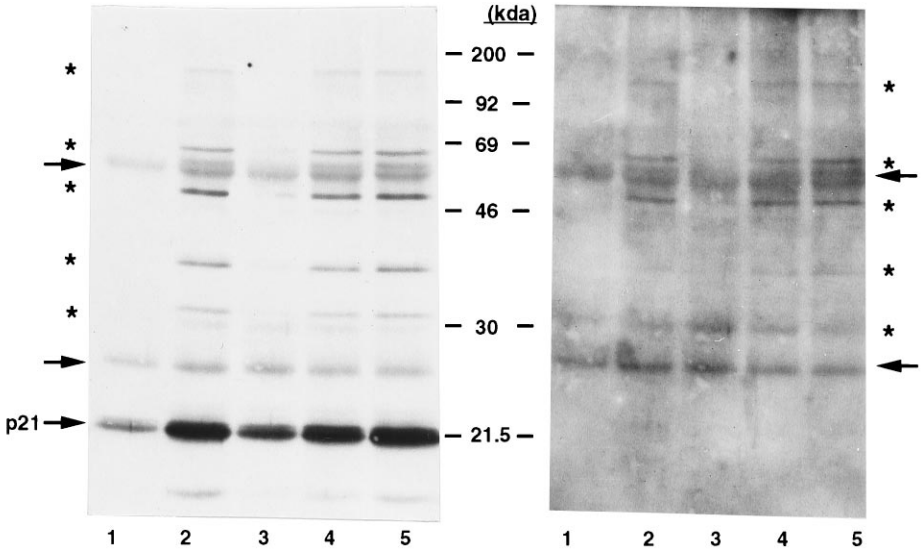

FIG. 5. Detection of p21-ubiquitin conjugates. U2OS cells were untransfected (lanes 1 and 2) or transfected with the ubiquitin expression plasmid pMT-123 (53) (lanes 3 to 5). The cells were then treated with 0.025 mM of the proteasome inhibitors MG115 (lanes 2 and 4) and lactacystin (lane 5) for 6 h, and protein extracts were prepared in RIPA buffer. p21 was immunoprecipitated from the extracts by using the 15431E p21 polyclonal antibody, and the immunoprecipitates were examined by Western blot analysis with the p21 monoclonal antibody 15091A (left). The blot was then stripped and reprobed with a ubiquitin-specific monoclonal antibody (right). The p21 band is indicated. Asterisks denote protein bands detected by both the p21 and ubiquitin antibodies. Background bands are indicated by arrows and are most likely due to detection of the antibody heavy chain (50 to 55 kDa) and light chain (25 to 30 kDa) used in the immunoprecipitation.

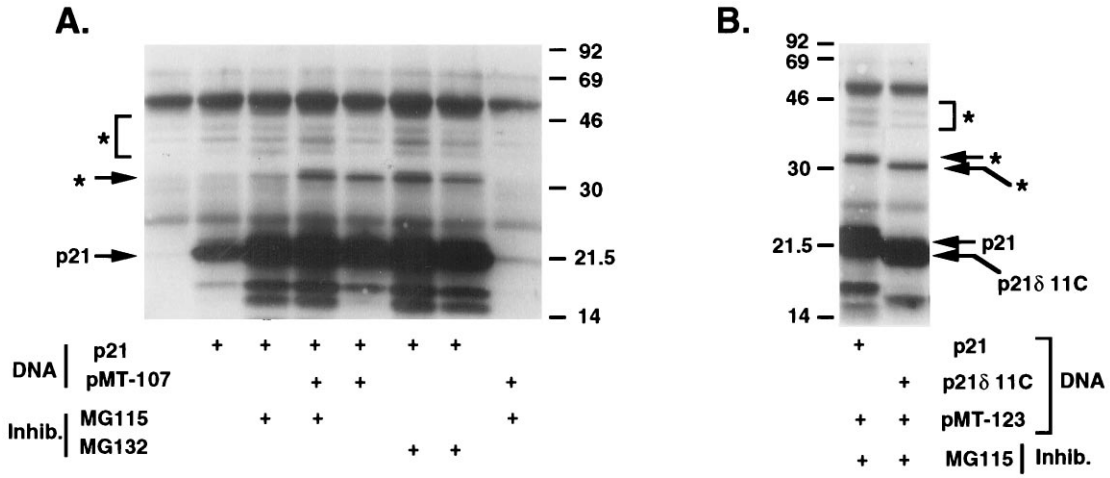

FIG. 6. Ubiquitination of p21 in Saos-2 cells transfected with a p21 expression plasmid. (A) Saos-2 cells were transfected with DNAs encoding p21 and the ubiquitin expression plasmid pMT-107 (53). Where indicated, cells were treated with the proteasome inhibitor (Inhib.) MG115 for 5 h or MG132 for 2.75 or 5 h. p21 was immunoprecipitated from transfected-cell extracts by using the 15431E p21 antibody and examined by Western blot analysis with the p21 monoclonal antibody 15091A. (B) Saos-2 cells were transfected with the ubiquitin expression plasmid pMT-107 (53) and full-length p21 or a p21 mutant with its C-terminal 11 amino acids deleted. The cells were treated with MG115 for 5 h, and p21 was immunoprecipitated and examined by Western blotting with the 15091A p21 antibody. The band marked with an asterisk at approximately 30 kDa in both blots appears to be ubiquitinated p21. The intensity of this band increases in response to proteasome inhibitors and in cells overexpressing ubiquitin. Higher-molecular-mass protein bands of ~40 to 50 kDa (also marked by asterisks) become more prevalent in cells coexpressing p21 and ubiquitin and treated with proteasome inhibitor. These bands may also be ubiquitinated forms of p21.

quently exposed to MG132, as in Fig. 7 (not shown). These results indicated that neither gamma nor UV irradiation causes an inhibition of cellular factors which ubiquitinate p21 and also indicated that the UV-induced loss of ubiquitination is therefore specific to p53. As shown in Fig. 7, increased steady-state levels of p21 were observed in gamma-irradiated cells (compare the zero time point, nonirradiated and gamma irradiated). This increase was probably due to increased levels of p53 activating p21 transcription. In contrast, p21 levels were not increased in the UV-irradiated cells, probably due to the dose of UV (20 J/m^2) used in these experiments. Perry et al. examined the effect of UV radiation on the ability of p53 to activate transcription of a responsive gene and demonstrated that the transactivation activity of p53 is transiently inhibited in cells treated with a UV dose of 19 J/m^2 (45). More recently, Lu et al. (33) also reported that p53 transcriptional activity is diminished with increasing UV doses. The diminished levels of p21 that we observe after UV treatment are consistent with an inhibition of the transcriptional activation function of p53.

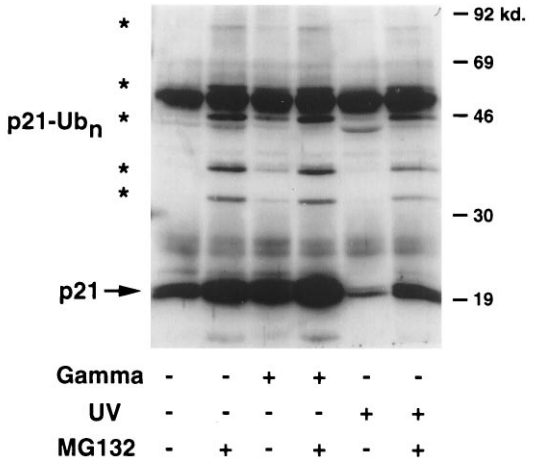

FIG. 7. Detection of ubiquitinated p21 in irradiated cells. U2OS cells were either untreated, treated with the proteasome inhibitor MG132 (0.025 mM), or treated with gamma (10-Gy) or UV (20-J/m^2) irradiation as indicated. When irradiation was combined with MG132 treatment, the cells were first irradiated and 1 h after the irradiation were treated with MG132 for an additional 5 h. When cells were treated singly with irradiation or MG132, cell lysates were prepared 6 h after each treatment. p21 was immunoprecipitated from the extracts using the 15431E p21 polyclonal antibody, and the immunoprecipitates were examined by Western blot analysis with the p21 monoclonal antibody 15091A. The p21 band is indicated. Asterisks denote ubiquitinated p21 species.

p21 protein stability is unaffected by irradiation. Since p21 can be ubiquitinated, we next examined the effects of gamma and UV irradiation on the stability of the p21 protein. The half-life of p21 protein was determined in cells which either were not irradiated or were irradiated under conditions which resulted in the stabilization of p53 (Fig. 8). The half-life of p53 in nonirradiated U2OS cells was approximately 30 min (Fig. 8A), and the half-life of p21 in nonirradiated U2OS cells was between 30 and 60 min (Fig. 8B). The half-life of p21 remained unchanged in U2OS cells in response to either gamma or UV radiation treatment, whereas both gamma and UV radiation resulted in the stabilization of p53. The p21 protein half-life was also unaffected in RKO cells which had been irradiated under conditions which resulted in the stabilization of p53 (data not shown). These results indicate that the irradiation-induced stabilization of p53 is not a property shared by all proteins which are degraded by the ubiquitin system.

DISCUSSION

p53 levels increase in cells exposed to a variety of DNA-damaging agents, including UV and gamma radiation. However, the mechanisms by which different types of radiation cause an increase in p53 levels are not understood. Cells from patients with the cancer-prone, radiation-sensitive disorder ataxia-telangiectasia (AT) have either a defective or a delayed induction of p53 in response to IR (4, 27, 29, 37, 38). It is therefore believed that the gene product that is defective in AT lies upstream of p53 in an IR-responsive signal transduction

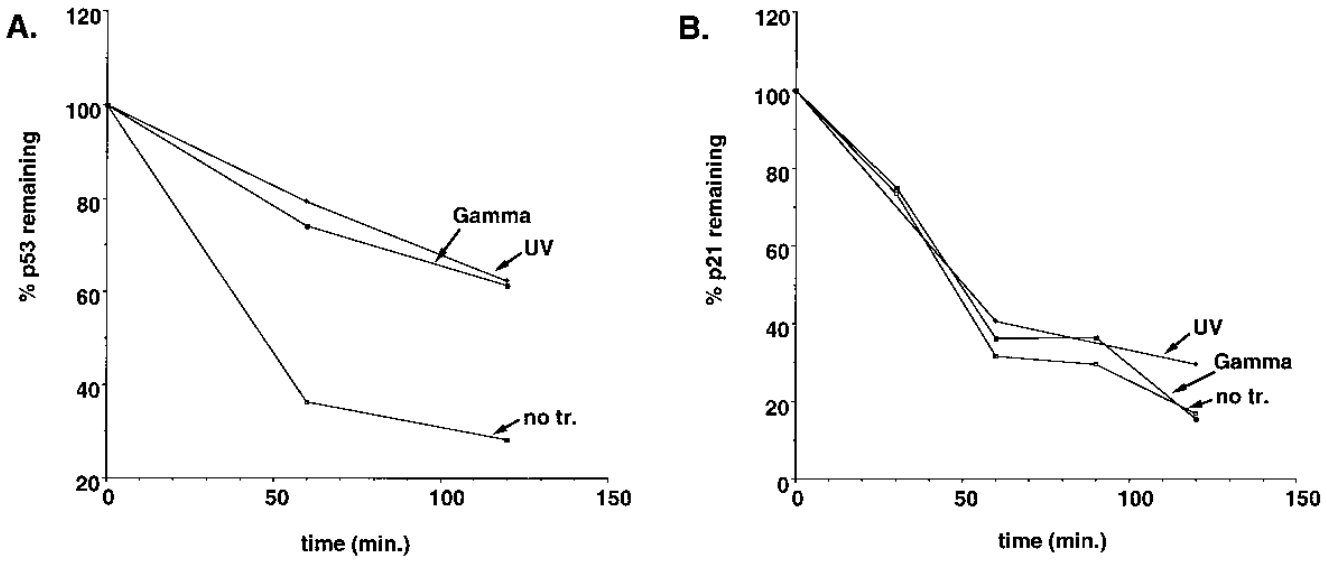

FIG. 8. p21 stability is unaffected following irradiation. U2OS cells were either nonirradiated (no tr.) or irradiated with gamma (10-Gy) or UV (10-J/m^2) irradiation. At 5 h after irradiation treatment, cycloheximide was added to a final concentration of 25 μg/ml. At the indicated times after addition of cycloheximide, cell lysates were prepared and examined by Western blot analysis for p53 and p21. The levels of p53 (A) and p21 (B) at each time point were quantitated by densitometric scanning of the resulting autoradiograms. Similar results were obtained in RKO cells (not shown).

pathway. Inhibitors of protein kinase C and phosphatidylinositol kinase (40, 46) have been reported to block an irradiation-induced increase in p53, implicating a role for distinct signaling pathways in the p53 response to radiation. Interestingly, the induction of p53 is not impaired in AT cells following UV irradiation (29, 37, 38). These findings suggest that IR and UV radiation work through different signaling pathways to cause an increase in the level of p53. The results presented in this paper demonstrate additional aspects of the p53 responses to gamma and UV radiation that are clearly different, further implicating different pathways in signaling these responses.

The most striking finding from our current study is that gamma radiation and UV radiation have different effects on the in vivo ubiquitination of p53. Both gamma and UV radiation resulted in a stabilization of the p53 protein. Ubiquitin-conjugated forms of p53 were detectable in nonirradiated and gamma-irradiated cells, and the levels of ubiquitinated p53 increased following gamma radiation treatment. In contrast, UV irradiation resulted in the rapid disappearance of ubiquitinated p53 species. These results indicate that the increased levels and stabilization of p53 observed following UV treatment could be a direct result of a loss of p53 ubiquitination.

There are several mechanisms by which p53 ubiquitination could be lost following UV treatment. First, it is possible that p53 is modified following UV irradiation in such a way that it is no longer recognized by the components of the ubiquitin system involved in its ubiquitination in normal cells. It has been reported that kinases which can phosphorylate p53 are activated in response to UV irradiation and that the pattern of p53 phosphorylation is altered following UV irradiation (39). Changes in the phosphorylation of p53 following UV irradiation could modify p53 in such a way that it can no longer be recognized by the ubiquitin-conjugating enzymes and the ubiquitin protein ligase involved in its ubiquitination. Second, UV radiation may inhibit the ubiquitination enzymatic machinery specific for p53. Our results with p21 indicate that the UV-mediated loss of ubiquitination is not global and is specific for p53. Third, UV irradiation could activate or stabilize one or more proteins that can deubiquitinate p53. While several deubiquitinating enzymes (also called isopeptidases) have been identified, to our knowledge the effect of irradiation on their activity has not been examined. It is interesting to note that p53 can be efficiently ubiquitinated and degraded following UV irradiation of cells that express the human papillomavirus E6 oncoprotein (32, 53). This may suggest that the cellular factors involved in the E6- and E6-AP-mediated ubiquitination of p53 are different from those involved in its ubiquitination in normal cells.

It is clear from our results that UV radiation and gamma radiation have very different effects on ubiquitinated forms of p53. The stabilization of p53 protein following UV irradiation could be due to the specific UV-induced inhibition of p53 ubiquitination. Though the exact mechanisms by which this occurs are currently unknown, our study represents the first molecular explanation for how UV irradiation causes the stabilization of p53. Insofar as ubiquitinated p53 could still be detected in gamma-irradiated cells, the stabilization of p53 following gamma radiation treatment appears to occur through a different mechanism acting downstream of p53 ubiquitination. One possibility is that gamma irradiation inhibits the degradation of ubiquitinated p53 by the proteasome, without affecting any putative p53-specific deubiquitinating activity.

In this study, we also detected ubiquitinated forms of p21, a cyclin-dependent kinase inhibitor, in cells exposed to proteasome inhibitors. Ubiquitin conjugates were detected for both the endogenous p21 protein and a transfected p21. Previous studies in our laboratory have shown that inhibition of the proteasome results in the stabilization of both the endogenous and a transfected p21 protein (34a, 35). Taken together, these results prove unequivocally that p21 is a direct target of ubiquitin-mediated degradation. As such, p21 now joins a growing list of cell cycle regulatory proteins which are degraded by the ubiquitin system (5, 15, 20, 43, 51). Included among these are the *Saccharomyces cerevisiae* cdk inhibitor p40^{SIC1} (51) and the mammalian cdk inhibitor p27 (43). The ubiquitination and degradation of both p40 and p27 appear to be tightly linked to the cell cycle. It is unclear at this time whether p21 ubiquitination is similarly regulated. Low levels of ubiquitinated p21

were also detected in U2OS cells that had been gamma irradiated. These results indicated that detection of ubiquitinated p21 did not require treatment with a proteasome inhibitor. Increased steady-state levels of p21 were observed in the gamma-irradiated cells, probably due to increased levels of p53 activating p21 gene transcription. The ability to detect ubiquitinated p21 following gamma irradiation may simply reflect the overall increase in p21 steady-state levels in irradiated cells.

Given the effects of gamma and UV irradiation on the stability and ubiquitination of p53, we examined the effects of gamma and UV treatment on the stability and in vivo ubiquitination of p21. While UV treatment resulted in the loss of ubiquitinated forms of p53, ubiquitin-p21 conjugates were still detected in UV-irradiated cells, indicating that the effects of UV on the ubiquitination of p53 are specific. Furthermore, the stability of p21 was unaffected under gamma or UV irradiation conditions which caused the stabilization of p53. This demonstrates that the stabilization of p53 following radiation treatment is not a property shared by all ubiquitin system targets. Our results have clearly demonstrated that p53 ubiquitination is regulated differently in response to IR and UV irradiation. It is not yet clear whether these different mechanisms of p53 stabilization result in any differential functional consequences on p53 activities.

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