MOLECULAR AND CELLULAR BIOLOGY, Dec. 1993, p. 7222–7231 Vol. 13, No. 12
0270-7306/93/127222-10$02.00/0

The Ionizing Radiation-Induced Replication Protein A Phosphorylation Response Differs between Ataxia Telangiectasia and Normal Human Cells

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Received 12 May 1993/Returned for modification 15 June 1993/Accepted 27 August 1993

Replication protein A (RPA), the trimeric single-stranded DNA-binding protein complex of eukaryotic cells, is important to DNA replication and repair. Phosphorylation of the p34 subunit of RPA is modulated by the cell cycle, occurring during S and G_2 but not during G_1. The function of phosphorylated p34 remains unknown. We show that RPA p34 phosphorylation is significantly induced by ionizing radiation. The phosphorylated form, p36, is similar if not identical to the phosphorylated S/G_2 form. γ-Irradiation-induced phosphorylation occurs without new protein synthesis and in cells in G_1. Mutation of *cdc2*-type protein kinase phosphorylation sites in p34 eliminates the ionizing radiation response. The γ-irradiation-induced phosphorylation of RPA p34 is delayed in cells from ataxia telangiectasia, a human inherited disease conferring DNA repair defects and early-onset tumorigenesis. UV-induced phosphorylation of RPA p34 occurs less rapidly than γ-irradiation-induced phosphorylation but is kinetically similar between ataxia telangiectasia and normal cells. This is the first time that modification of a repair protein, RPA, has been linked with a DNA damage response and suggests that phosphorylation may play a role in regulating DNA repair pathways.

Replication protein A (RPA) is a three-subunit protein complex involved in many DNA processes, primarily functioning as a nonspecific single-stranded DNA-binding activity. The p70 subunit exclusively encodes the single-stranded DNA-binding ability (3, 35, 60), whereas the functions of p34 and p11 are still unknown. The three subunits are highly conserved in eukaryotes (3, 4, 16, 30). In the yeast *Saccharomyces cerevisiae*, all three RPA subunits are essential for cell viability (4). Modification of RPA p34 is cell cycle regulated by phosphorylation on serine residues at the G_1-S transition and throughout S and G_2 phases (12, 14), and the kinases phosphorylating RPA p34 in vivo are unknown.

RPA is used in the initiation and elongation stages of DNA replication, as exemplified in the simian virus 40 (SV40) model system. RPA is necessary for SV40 large-T-antigen-dependent *ori* unwinding, primosome assembly, and elongation (3, 17, 35, 40, 42, 54, 59, 60). A nonspecific DNA-binding protein, such as RPA, might be expected to be involved in DNA repair and recombination. Since purified yeast and human RPA stimulate strand exchange proteins (SEP1 and HPP-1, respectively) in in vitro strand exchange assays (29, 43), it seems likely that RPA is involved in DNA repair pathways. It has been demonstrated that antibodies directed against RPA inhibited excision repair in an in vitro system, and addition of purified RPA reconstituted repair synthesis (9).

Eukaryotic cells are vulnerable to ionizing radiation damage primarily because of the DNA double-strand breaks formed that are associated with cell lethality (5, 6, 19, 49). In eukaryotes, a double-strand break repair mechanism that is responsible for repairing lethal damage of ionizing radiation also appears to utilize genes that are part of a DNA recombination pathway (1, 20, 22, 28, 51). In addition, cells have a broad spectrum of responses to ionizing radiation, including new gene transcription, protein synthesis, changes in protein stability, and posttranslational modification. Thus, it appears that a complex variety of programmed responses including activation of DNA repair, cell cycle arrest, and apoptosis are invoked by ionizing radiation. Ionizing radiation has been shown to induce several protein kinases and early-response genes encoding transcription factors (NFκB, Fos, c-Jun, EGR1) (2, 26, 50, 55, 56). γ-Irradiated cells undergo delays in cell cycle progression, exhibited as G_1/S and G_2 blocks or an inhibition of DNA synthesis (11, 27, 46, 53, 58). For example, *S. cerevisiae rad9* mutants both are hypersensitive to γ irradiation and do not undergo G_2 arrest in response to DNA damage (27, 57, 58). Down regulation of cell cycle regulatory genes, such as the cyclin A, cyclin B, *cdc2*, and *cdc25* genes, has been proposed to be involved in the cell cycle arrest induced by ionizing radiation (10, 38, 44). Generally, the genes that integrate DNA repair with cell cycle progression in mammalian systems in response to ionizing radiation have yet to be identified. Delays at cell cycle checkpoints may allow for repair to proceed before either replication or segregation of damaged DNA.

Ataxia telangiectasia (AT) is a human autosomal recessive disease characterized by hypersensitivity to ionizing radiation, increased predisposition to cancer, chromosome instability, and immune dysfunction (23). The hypersensitivity to ionizing radiation has been postulated to occur because of an inability to recognize a cell cycle block in G_1/S, S, or G_2/M (41, 46, 61, 62). For example, AT cells fail to stop DNA synthesis in response to γ irradiation (46). Cultured AT cells can repair X-ray-induced double-strand breaks by in vitro neutral filter elution but show altered properties in other short-term DNA repair and replication assays (reviewed in reference 41). Thus, the AT main defects have been suggested to be in the signaling of DNA repair.

In this study, we demonstrate that RPA phosphorylation in the p34 subunit of the trimeric complex is rapidly induced by ionizing radiation. Conversion of the p34 subunit to a p36

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form is similar if not identical to RPA phosphorylation normally observed in S phase. Interestingly, the accumulation of RPA phosphorylation occurs at a slower rate in AT cells. This is the first example of modification of a DNA repair protein in response to ionizing radiation. RPA phosphorylation in response to DNA damage may modulate its function in repair complexes or effect its localization to repair sites.

MATERIALS AND METHODS

Chemicals. Mimosine (Aldrich Chemical Co.) was resuspended in phosphate-buffered saline (PBS) at 10 mM. Emetine (Sigma) was resuspended in water at 20 mM. Hydroxyurea (Sigma) was resuspended in water at 1 M.

Cell culture and labeling. Raji, Daudi, and HL60 cells were grown with RPMI 1640 containing 10% fetal calf serum (FCS), 5% penicillin-streptomycin (P-S), and L-glutamine. GM08436, GM01526, and GM03189 cells (AT cells; NIGMS Human Genetic Cell Repository) and HPB-ALL cells were grown in RPMI 1640 containing 10% heat-inactivated FCS, 5% P-S, and L-glutamine. NIH 3T3 cells were grown in Dulbecco modified Eagle medium containing 10% newborn calf serum, 5% P-S, and L-glutamine. RP-9 and 23-11 cells were obtained from A. Dutta and grown in Dulbecco modified Eagle medium containing 400 μg of G418 per ml, 10% FCS, 5% P-S, and L-glutamine. To synchronize Raji cells in early G_1, exponentially growing cells (5×10^5 to 1×10^6 cells per ml) were treated with 400 μM mimosine for 12 h. Fluorescence-activated cell sorting (FACS) analysis was conducted on mimosine-blocked cells by propidium iodide staining and cell sorting to demonstrate that these cells have a G_1 DNA content (2N). GM08436 and Raji cells were synchronized in G_1/S by treating exponentially growing cells with 2.5 mM hydroxyurea for 12 h. FACS analysis was conducted on hydroxyurea-blocked and released Raji cells to demonstrate that these cells have a G_1/S DNA content and that the block is reversible. Protein synthesis was inhibited by treating Raji cells with 2 μM emetine for 15 min prior to γ irradiation.

Exponentially growing cells (5×10^6) were labeled with 0.25 mCi of [^{35}S]methionine (Translabel) (ICN) for 5 h in 3 ml of RPMI 1640 containing 1.5 mg of L-methionine per ml and 10% (vol/vol) dialyzed FCS. Cells (2×10^7) were starved in 0.5 ml of phosphate-free RPMI 1640 containing 10% (vol/vol) dialyzed FCS for 1 h. Immediately following γ irradiation, the cells were labeled with 0.2 mCi of $^{32}P_i$ (NEN) for 2 h. For phosphopeptide mapping, 5×10^7 cells were labeled for 3 h with 1 to 5 mCi of $^{32}P_i$ (NEN) either after exposure to 50 Gy or after 1 h of release into fresh medium subsequent to a 12-h block in hydroxyurea.

γ irradiation and UV irradiation. Irradiation with γ rays was carried out with a ^{60}Co source (Harvard School of Public Health; courtesy of John Little) at a dose rate of 13.5 rads/s. Cells were irradiated in media. Similar results were obtained whether cells were labeled with [^{35}S]methionine (Translabel) before or after γ irradiation. UV treatment of cells was carried out in 100-mm-diameter dishes in a Stratalinker (Stratagene) at a dose of 4,000 μW/cm^2.

Immunoprecipitation. Monoclonal antibodies against p70 and p34 subunits of RPA (p70-9, p70-16, and p34-20) were generously provided by Bruce Stillman, Salah-u-Din, and Anindya Dutta (Cold Spring Harbor Laboratory). Extracts were made from 5×10^6 cells by washing them in PBS and then lysing them in 50 mM Tris-Cl (pH 7.4)–250 mM NaCl–0.1% Nonidet P-40–5 mM EDTA. This buffer also contains 50 mM NaF, 1 mM sodium orthovanadate, 20 μM phenylarsine oxide, 2 mM phenylmethylsulfonyl fluoride, 0.7 μg of pepstatin per ml, 0.5 μg of leupeptin per ml, and 0.2 μg of aprotinin per ml (Sigma). All steps were performed at 4°C. RPA antibodies (50 μl) were incubated with labeled extract, adjusted to 400 μl, and kept on ice for 30 min. The immune complexes were precipitated with 1 μl of rabbit anti-mouse antibody (DAKO) and 50 μl of protein A-Sepharose (Pharmacia) and washed five times with the lysis buffer without the protease and phosphatase inhibitors. Some immunoprecipitates were treated with 1 U of calf intestinal alkaline phosphatase (Boehringer Mannheim) for 15 min at 37°C in 50 mM Tris (pH 8.0)–5 mM $MgCl_2$–25 mM NaCl. After the beads were boiled in 40 μl of sample buffer for 5 min, the proteins were loaded onto sodium dodecyl sulfate (SDS)–10% polyacrylamide gels. Protein molecular weight size standards were used in every gel.

Gel quantitation. ^{35}S-labeled immunoprecipitation products were analyzed by exposure on X-ray film. Densitometer scanning (Pharmacia LKB 2400 Gelscan) was completed to assess the ratio of p34 and p36 forms as noted in Results. The total amount of RPA p34 was not quantitated.

Immunoblotting. Cells (10^6) lysed in 1× Laemmli sample buffer were fractionated on SDS–10% polyacrylamide gels and transferred to nitrocellulose in 25 mM Tris–192 mM glycine–20% methanol at 40 mA for 2 h at 4°C. The following steps were all performed at room temperature. Nonspecific protein binding was blocked with 3% bovine serum albumin (BSA) in PBS for 1 h. The filters were probed with the antibodies (p34-20 diluted 1:3 in PBS–1% BSA) for 1 h, washed four times for 5 min each time with PBS–0.1% Tween 20, and incubated for 1 h with 0.2 μg of anti-mouse immunoglobulin G-alkaline phosphatase conjugate (Promega) per ml in PBS–1% BSA. After four 5-min washes with PBS–0.1% Tween 20, the blot was incubated in developing solution (150 μg of bromochloroindolyl phosphate per ml and 312 μg of nitroblue tetrazolium per ml [Promega] in 100 mM NaCl–5 mM $MgCl_2$–100 mM Tris [pH 9.5]). The reaction was stopped by washing the filter in water.

Phosphopeptide mapping. $^{32}P_i$-labeled RPA was immunoprecipitated from cell extracts and fractionated preparatively by SDS-polyacrylamide gel electrophoresis (PAGE). ^{32}P-labeled RPA p36 was extracted from stained, destained, and dried polyacrylamide gels for phosphopeptide mapping as described by Tang and Neel (51a) as follows. ^{32}P-labeled RPA p36 was excised and fixed in 1 ml of 30% methanol for three times over a period of 1 h and then overnight at 37°C. The gel slice was dried and digested with 0.1 mg of trypsin (tolylsulfonyl phenylalanyl chloromethyl ketone [TPCK] treated; Worthington) per ml in 500 μl of 50 mM ammonium bicarbonate overnight at 37°C. Two hours into the incubation, the digestion mixture was supplemented with an additional 200 μl of the trypsin solution. The samples were centrifuged for 10 min, and the supernatant was transferred to a new tube and dried. The dried sample was washed sequentially in decreasing volumes of water (1,000, 500, 300, 200, and 100 μl) and finally resuspended in 2 μl of 4.73 mM β-mercaptoethanol for loading. Phosphopeptides present in the supernatant were analyzed as described by Pines and Hunter (48) except that electrophoresis was at 1 kV for 25 min. Chromatography was in butanol-acetic acid-pyridine-water, 75:15:50:60 (vol/vol). In typical experiments, 500 to 3,000 cpm of ^{32}P-labeled RPA peptides was analyzed. Radioactive peptides were detected by autoradiography.

In vitro phosphorylation of RPA p34 with cyclin B-*cdc2* kinase. Purified RPA and cyclin B-*cdc2* kinase were gener-

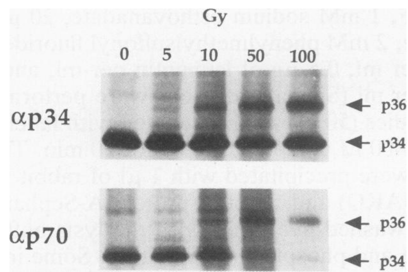

FIG. 1. γ-ray induction of a 36-kDa form of RPA. [^{35}S]methionine-labeled HPB-ALL cells were subjected to increasing doses of γ irradiation (0 to 100 Gy; see Materials and Methods). Cell lysates were prepared 2 h after γ-ray treatment, and RPA was immunoprecipitated with monoclonal antibodies specific to two of the subunits. Anti-p34 (αp34; p34-20) and anti-p70 (αp70; p70-9) each immunoprecipitate RPA by association of the 34- and 70-kDa subunits, respectively. Immunoprecipitates were then subjected to electrophoresis in an SDS–10% polyacrylamide gel and autoradiography. The p34 region of the SDS gel is shown for both p34-20 and p70-9 immunoprecipitations. Size standards were run with the gel to determine protein molecular weights (not shown).

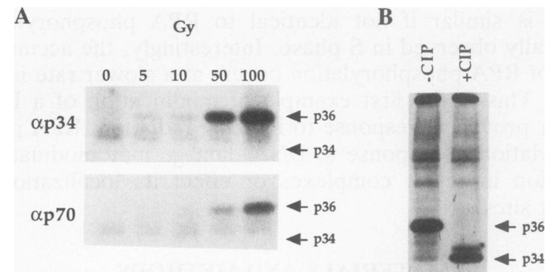

FIG. 2. Phosphorylation of the RPA p34 subunit following ionizing radiation. (A) HPB-ALL cells were treated with increasing doses of γ irradiation (0 to 100 Gy) and $^{32}P_i$ labeled for 2 h. RPA was immunoprecipitated with monoclonal antibody p34-20 (αp34) or p70-9 (αp70) and then subjected to SDS-PAGE (10% polyacrylamide gel) and autoradiography. (B) [^{35}S]methionine-labeled HPB-ALL cells were prepared as described in the legend to Fig. 1 and in Materials and Methods. Following immunoprecipitation with p70-16, the immunobeads either were not treated or were treated with 1 U of calf intestinal phosphatase (CIP) (Boehringer Mannheim) for 15 min at 37°C. The p70 subunit is shown at the top of the gel and is unaffected by CIP treatment.

ously provided by A. Dutta and B. Stillman. An in vitro kinase reaction was carried out for 20 min at 30°C, using 50 ng of purified RPA and 2 μl of purified cyclin B-*cdc2* kinase in 100 μl of kinase buffer (50 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 1 mM dithiothreitol, and 10 μM ATP containing 150 μCi of [γ-^{32}P]ATP). RPA was immunoprecipitated with 50 μl of monoclonal antibody p34-20 and analyzed by SDS-PAGE.

RESULTS

Ionizing radiation induces phosphorylation of the p34 subunit of RPA. RPA p34 is phosphorylated at the G_1-S transition, resulting in a 2-kDa increase in size (12). We reasoned that phosphorylation of RPA may also be modulated in response to γ irradiation. [^{35}S]methionine-labeled HPB-ALL human T cells were subjected to increasing doses of γ irradiation, and RPA was examined by immunoprecipitation with monoclonal antibody p34-20. A higher-molecular-weight form of RPA p34 accumulated in a dose-dependent manner (p36; Fig. 1). Two hours post-γ irradiation, the p36 form constituted 1/10 of the total RPA in the cells treated with 10 Gy and 1/3 of the total RPA with treatment at 50 or 100 Gy, determined by densitometry. Similar results were seen by immunoblotting with p34-20, demonstrating that a significant fraction of the total RPA p34 becomes phosphorylated by ionizing radiation. The conversion to the p36 form was also evident between 15 and 45 min after irradiation but to a lower extent (see Fig. 7). The γ-irradiation-stimulated p36 was also produced in several different transformed human lymphoid cell lines (Raji, Daudi, and Namalwa) and several transformed human and monkey tumor cell lines (HL60, SV80, CVIP, HeLa, and 293) (data not shown), suggesting that the conversion of p34 to p36 is generalizable and independent of cell type. As expected, the radiomimetic agent bleomycin also induced RPA p34 phosphorylation (data not shown). We observed no ionizing radiation-induced electrophoretic changes in the other two subunits of the RPA complex (p70 and p11) (data not shown).

Because a p36 form was previously associated with RPA phosphorylation during normal cell growth (12), we examined whether the p36 form induced by γ irradiation was phosphorylated. HPB-ALL cells were treated with γ rays, pulsed with $^{32}P_i$, and then subjected to RPA immunoprecipitation with monoclonal antibody p34-20 or p70-9. We observed a 36-kDa phosphorylated protein that accumulated with γ irradiation in a dose-dependent manner but did not appear in unirradiated cells (Fig. 2A). No incorporation of $^{32}P_i$ into a 34-kDa protein was detected by this method, indicating that any phosphorylation changes the mobility of the p34 subunit. Also, no phosphorylation of the p70 and p11 subunits was found (data not shown). Second, lysates from γ-irradiated [^{35}S]methionine-labeled HPB-ALL cells were immunoprecipitated with monoclonal antibody p70-16, and the precipitates were treated with calf intestinal phosphatase to remove phosphate groups on the protein (Materials and Methods). p36 was efficiently converted to p34 (Fig. 2B), also suggesting that the p36 is a phosphorylated form of p34.

p34 is found in the RPA holocomplex or as free p34 monomers; these forms can be distinguished by differential precipitation analysis with monoclonal antibodies p70-9 and p34-20. The RPA complex is the target for p34 phosphorylation during S and G_2 phases (14, 18). We determined whether the transition to the phosphorylated form (p36) occurred in the RPA complex or as free p34 in response to γ irradiation for the [^{35}S]methionine-labeled cells. Immunoprecipitation with p70-9 yields p34 from RPA, whereas p34-20 precipitates both the complexed and free forms of p34. A greater fraction of RPA p34 (75%) was in the phosphorylated form from p70-9 immunoprecipitation than seen with p34-20 immunoprecipitation (33%), as determined by densitometry (Fig. 1). Thus, following sufficient γ irradiation, most of the p34 phosphorylation occurs in RPA and the majority of RPA complexes have been hyperphosphorylated.

Extensive phosphorylation on serine residues in an NH_2-terminal region of p34 was observed in S and G_2 phases (14). We used several criteria to test whether the γ-irradiation-stimulated p34 phosphorylation was similar to this S/G_2 phase form. Raji cells were treated with hydroxyurea to block the cells in early S phase. FACS analysis of cells treated with hydroxyurea and subsequently released into fresh medium confirms that the hydroxyurea-blocked cells have a G_1/S DNA content and that the block is reversible (Fig. 3B). The p36(S/G_2) and p36(γ ray) proteins comigrate

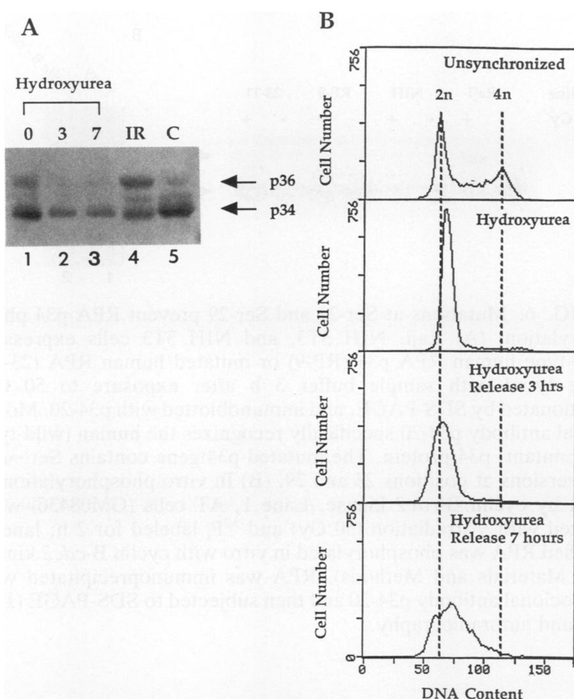

FIG. 3. Comparison of p36(S phase) and p36(γ ray). (A) Lysates were made from Raji cells treated as follows: lane 1, 2.5 mM hydroxyurea for 12 h; lane 2, 2.5 mM hydroxyurea for 12 h and released into fresh medium for 3 h; lane 3, 2.5 mM hydroxyurea for 12 h and released into fresh medium for 7 h; lane 4, exponentially growing cells lysed 3 h after treatment with γ irradiation (50 Gy); and lane 5, exponentially growing cells untreated. All cells were lysed in sample buffer and fractionated by SDS-PAGE (10% polyacrylamide gel) prior to Western blotting with p34-20. (B) FACS analysis of cells stained with propidium iodide with and without hydroxyurea treatment (2.5 mM) and at 3 and 7 h following release from hydroxyurea treatment (see Materials and Methods).

by electrophoretic mobility in SDS-PAGE and Western blot (immunoblot) analysis (Fig. 3A). p36 is most pronounced in hydroxyurea-blocked cells and diminishes as cells progress through S phase.

We used phosphopeptide mapping to determine whether the two p36 forms could be distinguished. ^{32}P-p36(S/G_2) and ^{32}P-p36(γ ray) were isolated from Raji cells and compared by exhaustive tryptic digestion and two-dimensional electrophoresis–thin-layer chromatography (Materials and Methods). Four unique phosphopeptides (A to D) were discernible (Fig. 4A and B). The patterns and relative intensities of phosphopeptides A to D were the same for p36(S/G_2) and p36(γ ray), a further indication that these forms are identical. In addition, a phosphoamino acid analysis of isolated p36(γ ray) indicated that phosphorylation occurs on serine residues exclusively, with no detectable phosphothreonine or phosphotyrosine residues (data not shown). Similarly, using an antiphosphotyrosine antibody (4G10; courtesy of Brian Drucker and Tom Roberts) to probe Western blots of p34-20 immunoprecipitates from irradiated cells, we did not detect any p34 tyrosine phosphorylation in response to ionizing radiation. Therefore, p36 has extensive phosphoserine modifications similar to those of the phosphorylated S/G_2 form.

RPA phosphorylation occurs in G_1 in response to γ rays. DNA repair responses may be activated in any cell cycle phase. Therefore, we next assessed whether RPA p34 phos-

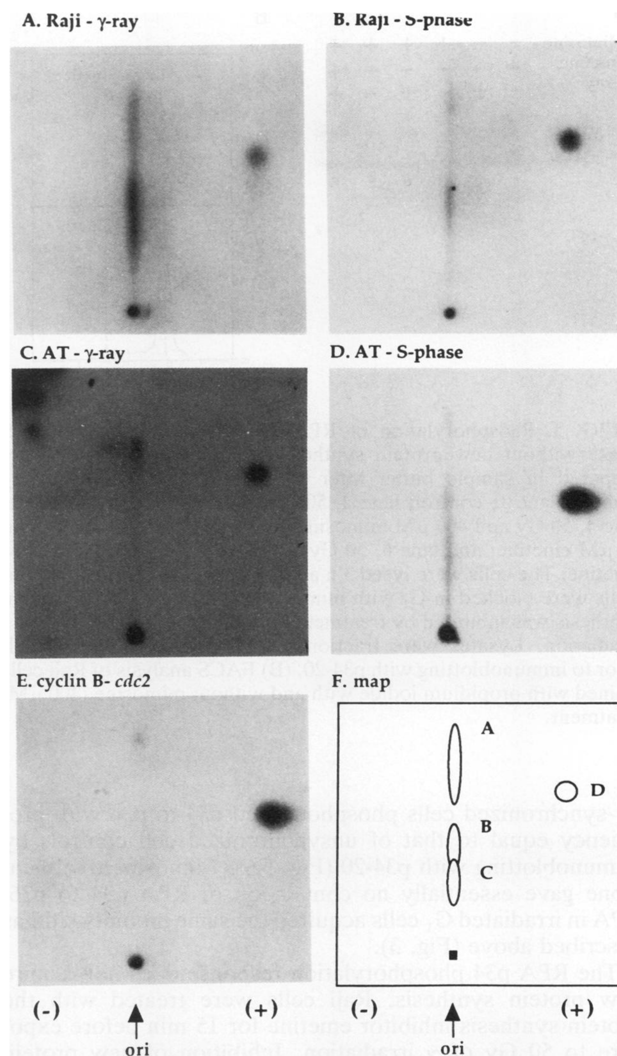

FIG. 4. Phosphopeptide maps of phosphorylated RPA p34. The peptides from trypsin-digested RPA were separated by electrophoresis and thin-layer chromatography (ascending). ^{32}P-RPA forms were isolated by immunoprecipitation (p34-20) from either normal (Raji) or AT (GM08436) cell lines grown in $^{32}P_i$ for 3 h or from in vitro-phosphorylated RPA p34 by purified cyclin B-*cdc2* kinase as described in Materials and Methods. (A) RPA p36 from Raji cells treated with γ irradiation (50 Gy). (B) RPA p36 from Raji cells treated with 2.5 mM hydroxyurea for 12 h and subsequently released into fresh medium for 1 h. (C) RPA p36 from AT cells (GM08436) treated with γ irradiation (50 Gy). (D) RPA p36 from AT cells (GM08436) treated with 2.5 mM hydroxyurea for 12 h and subsequently released into fresh medium for 1 h. (E) In vitro phosphorylation product of purified RPA with purified cyclin B-*cdc2* kinase (Fig. 6B). (F) Diagram showing the RPA p36 phosphopeptide map. Individual phosphopeptides were assigned letters A to D. ori, origin for electrophoretic dimension.

phorylation induced by ionizing radiation could occur in cells outside of S and G_2. The amino acid analog mimosine blocks mammalian cells in early G_1 (31, 36). Raji cells were synchronized by treatment with mimosine and demonstrated to be in G_1 by propidium iodide staining and FACS analysis (Materials and Methods) (Fig. 5B). The mimosine block is reversible, and the time of entry into S phase is consistent with a block in early G_1 (data not shown). We found that

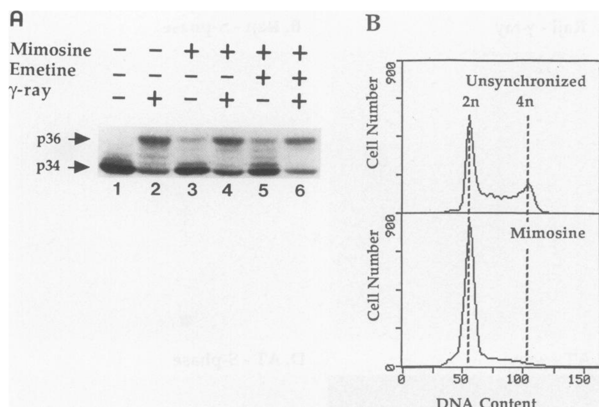

FIG. 5. Phosphorylation of RPA p34 can occur outside of S phase without new protein synthesis. (A) Raji cell lysates were prepared in sample buffer after being treated in the following manner: lane 1, control; lane 2, 50 Gy; lane 3, 400 μM mimosine; lane 4, 50 Gy and 400 μM mimosine; lane 5, 400 μM mimosine and 20 μM emetine; and lane 6, 50 Gy, 400 μM mimosine, and 20 μM emetine. The cells were lysed 3 h after treatment with γ irradiation. Cells were blocked in G_1 with mimosine for 12 h, and new protein synthesis was inhibited by treatment with emetine 15 min prior to γ irradiation. Lysates were fractionated by SDS-PAGE (10% gel) prior to immunoblotting with p34-20. (B) FACS analysis of Raji cells stained with propidium iodide with and without mimosine (400 μM) treatment.

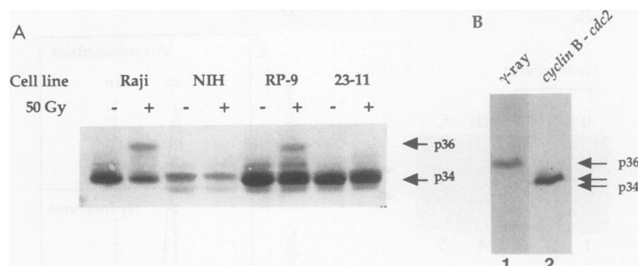

FIG. 6. Mutations at Ser-23 and Ser-29 prevent RPA p34 phosphorylation. (A) Raji, NIH 3T3, and NIH 3T3 cells expressing wild-type human RPA p34 (RP-9) or mutated human RPA (23-11) were lysed with sample buffer 3 h after exposure to 50 Gy, fractionated by SDS-PAGE, and immunoblotted with p34-20. Monoclonal antibody p34-20 specifically recognizes the human (wild-type and mutant) p34 protein. The mutated p34 gene contains Ser→Ala conversions at positions 23 and 29. (B) In vitro phosphorylation of RPA by cyclin B-*cdc2* kinase. Lane 1, AT cells (GM08436) were treated with γ irradiation (50 Gy) and $^{32}P_i$ labeled for 2 h; lane 2, purified RPA was phosphorylated in vitro with cyclin B-*cdc2* kinase (see Materials and Methods). RPA was immunoprecipitated with monoclonal antibody p34-20 and then subjected to SDS-PAGE (10% gel) and autoradiography.

G_1-synchronized cells phosphorylated p34 to p36 with proficiency equal to that of unsynchronized cell controls by immunoblotting with p34-20 (Fig. 5A). Mimosine treatment alone gave essentially no conversion of RPA p34 to p36. RPA in irradiated G_1 cells acquired the same mobility shift as described above (Fig. 3).

The RPA p34 phosphorylation response does not require new protein synthesis. Raji cells were treated with the protein synthesis inhibitor emetine for 15 min before exposure to 50 Gy of γ irradiation. Inhibition of new protein synthesis by emetine has no effect on the ability to phosphorylate p34 in response to γ irradiation (Fig. 5A). Nearly 50% of the total cellular p34 is converted to p36. In addition, mimosine-synchronized cells were also blocked for new protein synthesis with emetine. These cells are equally capable of phosphorylating p34 to the p36 form following γ irradiation (Fig. 5A). These studies suggest that RPA phosphorylation is a general DNA damage response that is not restricted to S phase where RPA is proposed to function. Also, the response is rapid and occurs on preexisting RPA complexes, because there is no requirement for new protein synthesis.

***cdc2* p34 kinase sites are required for γ-irradiation-induced phosphorylation.** *cdc2* kinases are important regulators of entry into S phase and the G_2/M transition (13, 21, 24, 25, 45). RPA phosphorylation occurring in S and G_2 is controlled by activation of *cdc2* kinases (14). To evaluate the importance of these *cdc2* kinase phosphorylation sites for the γ-ray-inducible response, we have used mutant versions of p34 prepared previously (14).

p34 phosphorylation in vitro depends on the presence of Ser-23 and Ser-29 residues (14). Mutation of these two phosphorylation sites in a transfected human p34 gene inhibits phosphorylation of the expressed p34 protein produced during S phase (14). NIH 3T3 mouse cells that had been stably transfected with wild-type human p34 (RP-9) or the dual mutations (Ser to Ala-23 and Ser to Ala-29) (23-11) were treated with 50 Gy. Cell lysates were prepared 3 h after irradiation and then inspected by SDS-PAGE and immunoblotting with monoclonal antibody p34-20. The transfected wild-type and mutant p34 genes are expressed constitutively in NIH 3T3 cells and at equivalent levels (Fig. 6A). The wild-type human p34 subunit is phosphorylated to the p36 form in response to γ irradiation in mouse cells. Mutation of Ser-23 and Ser-29 does not alter the mobility or the abundance of p34, and therefore p34 has not undergone complete phosphorylation to the p36 form (Fig. 6A). These effects are specific to the expressed human p34 because very little cross-hybridizing signal is observed for mouse p34 present in untransfected NIH 3T3 cells. These studies strongly suggest that phosphorylation of Ser-23 and Ser-29 is required for γ-ray-induced responses for RPA.

Four different phosphopeptides (A to D) were discernible by tryptic mapping (Fig. 4). To define which of these phosphopeptides can be attributed to cyclin B-*cdc2* kinase, purified RPA was phosphorylated in vitro with purified cyclin B-*cdc2* kinase, resulting in an intermediate migrating form between p34 and p36 (Fig. 6B). This phosphorylation corresponds to phosphopeptide D from tryptic mapping (Fig. 4E), suggesting that these two sites do not account for all p34 phosphorylation. However, their phosphorylation is presumably a prerequisite for additional γ-irradiation-induced phosphate addition and indicates an order in the phosphorylation pathway of p36 in response to DNA damage.

γ-ray induction of p34 phosphorylation is mediated by an AT gene product(s). AT cells exhibit deficiencies in recombination, repair, and replication following ionizing radiation damage. Because RPA has been associated with all three processes, we reasoned that AT cells may have altered RPA functions. Therefore, we examined RPA phosphorylation from several independently isolated cell lines from normal and AT patients. The amount of RPA p34 phosphorylation is both dose and time dependent in normal and AT cell lines (Fig. 7). p34 phosphorylation is detected by 45 min in normal cell lines following exposure to 50 Gy (HPB-ALL, Raji, Daudi, and HL60; Fig. 7A). At a lower dose, it takes a longer

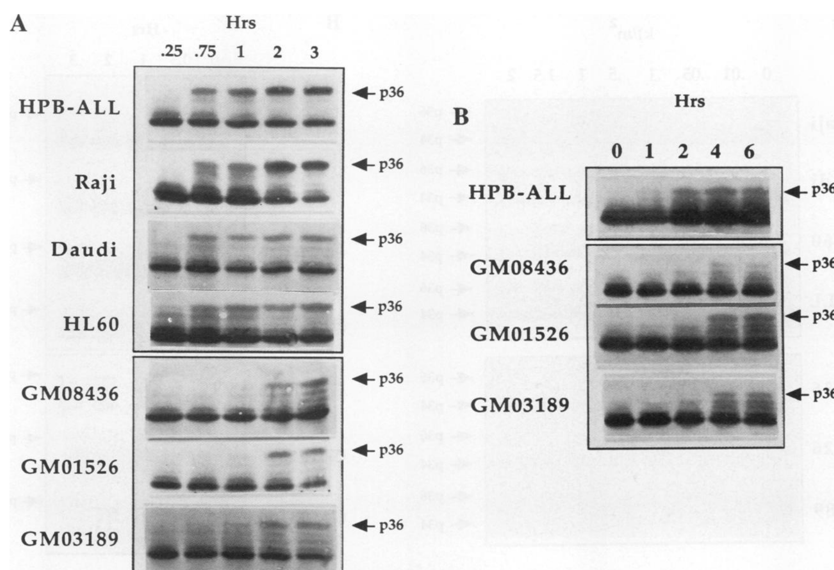

FIG. 7. Time course of RPA p34 phosphorylation in response to γ irradiation. (A) Western blot of RPA p34 prepared from four normal cell lines (HPB-ALL, Raji, Daudi, and HL60) and three AT cell lines (GM08436, GM01526, and GM03189) at 0.25 to 3.0 h after treatment with γ irradiation (50 Gy). (B) Western blot of RPA p34 prepared from 1 normal cell line (HPB-ALL) and three AT cell lines (GM08436, GM01526, and GM03189) at 0 to 6 h after treatment with γ irradiation (10 Gy).

time for p34 to become phosphorylated, and the p36/p34 ratio is less than that seen with higher doses of γ irradiation.

In contrast to the rapid p34 phosphorylation response seen in normal cells, AT cells are delayed in the ability to hyperphosphorylate p34 in response to γ irradiation. In AT cells, the hyperphosphorylated form of RPA p34 is not detected until 2 h after treatment with γ irradiation (GM08436, GM01526, and GM03189; Fig. 7A). At the lower dose of 10 Gy, p36 appearance is delayed to 2 to 4 h in the three AT cell lines (Fig. 7B). Although AT cells are slower in phosphorylating p34 in response to γ irradiation, the p36/p34 ratio detected at 3 h after γ-irradiation treatment is similar to that seen in normal cell lines at all doses (data not shown). These AT cell lines also show an increased level of p34 and p36 intermediate-size forms at the lower dose. p36 forms induced by γ rays in AT cells (GM08436) were shown to be of the same phosphorylation composition as p36(S/G_2) in AT (GM08436) and Raji cells and p36(γ ray) in Raji cells by tryptic phosphopeptide mapping (Fig. 4). GM01526 cells are in AT complementation group E, whereas the complementation groups of the other two AT cell lines are unknown. However, as patients from complementation group E are very rare (32), it seems likely that the other two AT cell lines fall into either complementation group A, C, or D. In addition, somatic cell fusion studies in our laboratory indicate that GM08436, GM01526, and GM03189 fall in different complementation groups (37a). Thus, the delayed p34 phosphorylation response is probably a shared phenotype among different AT complementation groups.

Conversion of p34 to p36 RPA in response to UV damage. A similar role for RPA activity may be expected in many types of single- and double-strand break repair. However, AT cells are hypersensitive to ionizing radiation and radiomimetic agents but not other DNA-damaging agents such as UV or alkylating agents (reviewed in reference 41). Therefore, we assessed RPA phosphorylation response to UV irradiation to determine how closely associated RPA phosphorylation is to an AT phenotype. Interestingly, UV damage also promoted p34 phosphorylation in normal cells, as detected by immunoblotting with p34-20 (Fig. 8). p34 phosphorylation was both dose and time dependent but accumulated less rapidly in the time course than for ionizing radiation (Fig. 8). The phosphorylated forms induced by either method comigrated on SDS-polyacrylamide gels (data not shown). We observed that AT and normal cells do not significantly differ in their p34 phosphorylation in either dose or time dependence in response to UV irradiation (Raji, Daudi, HL60, HPB-ALL, GM08436, GM01526, and GM03189; Fig. 8). In any of the cell lines tested, no phosphorylated RPA p34 was seen until 2 h following treatment with UV irradiation of 0.5 kJ/m^2. In response to increasing doses of UV, conversion of p34 to p36 becomes detectable at 0.05 kJ/m^2 and seems to be maximized at doses greater than 1 kJ/m^2. Lower sublethal UV doses consistently yielded lower conversion to p36 than did higher doses in each of the cell line tested.

DISCUSSION

We have shown that RPA p34 phosphorylation occurs in response to ionizing or UV radiation, causing an approximately 2-kDa size increase. Phosphorylation occurred in the RPA complex and was similar, if not identical, to S and G_2 RPA phosphorylation shown previously (14, 18). The γ-ray-inducible RPA modification is independent of new protein synthesis or cell cycle phase. The phosphorylation profile requires intact p34 Ser-23 and Ser-29 residues, sites of phosphorylation by *cdc2* kinases. We also show that the irradiation damage response of RPA p34 phosphorylation is present but is delayed in cell lines from AT patients specifically for ionizing radiation. These findings suggest that RPA phosphorylation may be a component of a DNA damage response associated with AT complementation groups.

Phosphorylation of RPA p34. p34 phosphorylation was observed to be dose dependent for ionizing radiation (Fig. 1, 2, and 7). Low sublethal doses converted p34 to the hyperphosphorylated form, though the p36/p34 ratio was lower

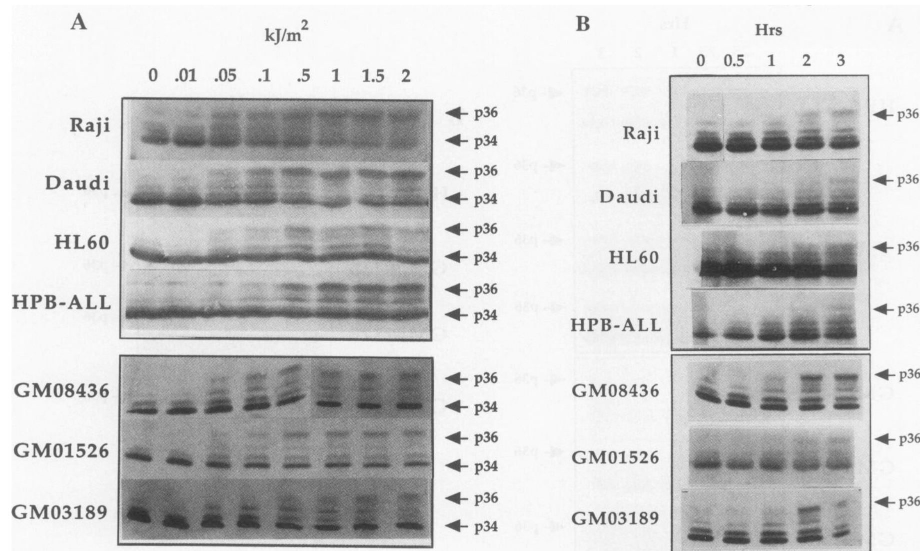

FIG. 8. Dose curve and time course of RPA p34 phosphorylation in response to UV irradiation. (A) Western blot of RPA p34 prepared from four normal cell lines (Raji, Daudi, HL60, and HPB-ALL) and three AT cell lines (GM08436, GM01526, and GM03189) treated with increasing doses of UV irradiation (0 to 2 kJ/m^2) at 3 h. (B) Western blot of RPA p34 prepared from four normal cell lines (Raji, Daudi, HL60, and HPB-ALL) and three AT cell lines (GM08436, GM01526, and GM03189) at 0 to 3 h after treatment with UV irradiation (0.5 kJ/m^2).

than at high doses (Fig. 1 and 2). It was interesting that only the hyperphosphorylated form, not intermediate forms of p34, was appreciably detected. This finding suggests that the dose determines the fraction of RPA to be phosphorylated rather than the extent of phosphorylation per p34 subunit. A small fraction of phosphorylated complexes may be indicative of the extent of formation of chromosomal DNA repair sites and may be linked to the number of double-strand breaks generated. Our experiments indicate that preexisting RPA is phosphorylated within 15 to 45 min following irradiation (Fig. 7). This rapid conversion of p34 to p36 is consistent with a direct effect signaled by ionizing radiation damage, rather than indirect effects that would be more likely to require longer times to accumulate.

Our results are suggestive that the damage-induced RPA phosphorylation is structurally similar to the accumulated p36 form during S phase. Extensive phosphorylation on multiple serines near the N terminus presumably generates the dramatic protein conformation shift that is reflected in reduced mobility on SDS-PAGE (12, 14, 18). p36(S/G_2), p36(γ ray), and p36(UV) comigrate on SDS-PAGE (Fig. 3A and data not shown). Phosphopeptide mapping of the p36 (S/G_2) and p36(γ ray) forms from both normal and AT cells gave maps that are indistinguishable (Fig. 4).

Our data are most supportive of a model whereby multiple kinases are being used to phosphorylate p34 in vivo. The mobility of the phosphorylated form on SDS-PAGE is intermediate (Fig. 5B), and cyclin B-*cdc2* kinase phosphorylation of RPA in vitro shows primarily phosphorylation of phosphopeptide D (Fig. 4E). Two of the same serine residues are needed for RPA p34 phosphorylation in response to γ irradiation or in S phase. Phosphorylation of these two residues does not account for all of the phosphorylation of p34, although little if any phosphate addition occurs when these sites are mutated (Fig. 6A).

Our results are consistent with a hypothesis that the same family of *cdc2*-type protein kinases that phosphorylate p34 in S/G_2 are also being recruited in a DNA damage response. Purified cyclin B-*cdc2* and cyclin A-*cdk2* kinases have previously been shown to phosphorylate RPA p34 in vitro (14, 15). However, these specific kinases may not be used in vivo, although it is very likely that one or more cyclin-dependent *cdk*-type kinase is used. A *cdc2* kinase may initially phosphorylate RPA at Ser-23 and Ser-29, leading to a protein structural change that makes the partially phosphorylated form a good substrate for other kinases. Different kinases may be regulated by ionizing radiation, depending on the expression of cyclins and kinases that are available at the cell cycle phase where DNA damage occurs. Alternatively, the same kinases that phosphorylate RPA in S phase may be recruited into DNA repair pathways at any cell cycle stage. In support of the second hypothesis, it is intriguing that ionizing radiation stimulates phosphorylation even though cells are in G_1. G_1-blocked cells (76% cells in G_1 by propidium iodide staining and sorting), G_1-synchronized cells (blocked and released but still in G_1), and asynchronous cells (approximately 38% in G_1) are active for kinases that will phosphorylate RPA p34 following ionizing radiation (Fig. 5). Therefore, RPA kinases may be constitutively present at all cell cycle phases but negatively regulated outside of S/G_2.

Interestingly, G_1 extracts are able to phosphorylate RPA p34 specifically when single-stranded DNA is added (18). The presence of single-stranded DNA may be functionally equivalent to broken DNA at repair sites. A DNA repair function for RPA could be analogous to the phosphorylation of RPA in the initiation of SV40 DNA synthesis. Phosphorylation of RPA in S-phase cell extracts requires SV40 T antigen and SV40 *ori*-containing plasmid DNA in vitro (18). T-antigen *ori* unwinding is thought to generate single-stranded DNA for RPA binding. p34 phosphorylation occurs following binding in vitro. RPA phosphorylation may be used for an additional function, such as to organize the initiation complex or for association with polymerase-primase rather than to facilitate DNA binding (18, 42). Similarly, RPA recruitment into a DNA repair site would occur whenever single-stranded DNA is formed by DNA damage, but the ability to drive RPA into repair complexes would be regulated by an ionizing radiation response.

AT gene products may be upstream of RPA phosphorylation in responses to ionizing radiation. AT cells are hypersensitive to ionizing radiation and exhibit several cell cycle defects, indicating that they are unable to process damage signals properly to invoke cell cycle blocks (41, 46, 61, 62). The p53 protein has recently been implicated as a G_1/S-phase checkpoint regulator for ionizing radiation damage responses and apoptosis (7, 33, 34, 37, 39). In response to ionizing radiation, p53 levels are increased for normal cells. AT cells apparently do not increase p53 levels, implying that AT genes may regulate the stability of p53 normally or affect the p53 half-life following irradiation (33, 34). AT cells have an inability to detect cell cycle irradiation checkpoints in G_1, S, and G_2 phases, arguing that all AT effects are unlikely to be mediated through p53.

Our experiments also associate AT gene products in DNA repair, since the rates of RPA phosphorylation are affected by AT mutations. We found that RPA phosphorylation was delayed in response to ionizing radiation in three AT lymphoblastoid cell lines (Fig. 7). The p36 form was generated in AT cells to a greater extent than underphosphorylated intermediates. Thus, AT cells possess the correct kinases for RPA phosphorylation, but the regulation of this phosphorylation pathway is disturbed. HL60 cells, which do not express p53, are as capable as normal cells in phosphorylating RPA p34 in response to ionizing radiation (Fig. 7A). It is interesting that normal and AT cells have similar induction of RPA p36 by UV irradiation (Fig. 8), arguing that the AT-specific DNA damage phenotypes are more tightly associated with double-strand break repair. Because p34 phosphorylation occurs in any cell cycle phase and independently of p53 expression, AT gene products must affect both p53-dependent and p53-independent repair responses. It is not known whether the AT mutations are null or leaky alleles, because these genes have not yet been cloned. Partially active or conditional AT alleles may explain the delayed ability to phosphorylate p34 following γ irradiation, whereas null alleles may have more complete effects on RPA phosphorylation.

AT cell extracts support SV40 DNA replication in vitro, suggesting that the RPA phosphorylation required to initiate DNA synthesis is intact in these mutant cell extracts (37a). If the same kinases are used for DNA replication and damage-induced RPA phosphorylation, it is reasonable to argue that AT cells do contain the appropriate kinases which phosphorylate RPA once it is bound to DNA. Therefore, an alternate model that could explain delayed phosphorylation in AT in response to ionizing radiation may be that the accessibility of RPA to repair sites, or binding at these sites, is more rate limiting. AT cell lines have been demonstrated to have altered chromatin properties from normal cells following irradiation, by using premature chromatin condensation techniques (8, 47). Also, fluctuations in chromatin structure as measured by supercoiling and unwinding assays of chromatin loop sizes following γ irradiation differ from those of normal cells (52).

Insight into the role of protein phosphorylation in modulating DNA repair responses may be instrumental in further understanding the nature of AT defects and the regulation of responses to ionizing radiation.

ACKNOWLEDGMENTS

We are indebted to the Stillman laboratory (Cold Spring Harbor Laboratory) for helpful discussions, advice, use of reagents, and the generous donation of RPA monoclonal antibodies p34-20, p70-9, and p70-16. We also thank A. Dutta and B. Stillman for providing NIH 3T3 RP-9 and 23-11 cells, purified RPA p34, and cyclin B-*cdc2* kinase. We thank V. Rivera, M. Greenberg, T. Tang, and B. Neel for assistance with phosphoamino acid mapping and phosphopeptide analysis. We thank Sue Brogna for the photography work. We thank members of the Weaver laboratory, A. Dutta, and T. Orr-Weaver for critically reading the manuscript and many helpful suggestions.

This work was supported by a grant from Sandoz Pharmaceuticals to D.T.W.

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