UV-induced Hyperphosphorylation of Replication Protein A Depends on DNA Replication and Expression of ATM Protein

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Exposure to DNA-damaging agents triggers signal transduction pathways that are thought to play a role in maintenance of genomic stability. A key protein in the cellular processes of nucleotide excision repair, DNA recombination, and DNA double-strand break repair is the single-stranded DNA binding protein, RPA. We showed previously that the p34 subunit of RPA becomes hyperphosphorylated as a delayed response (4–8 h) to UV radiation (10–30 J/m^2). Here we show that UV-induced RPA-p34 hyperphosphorylation depends on expression of ATM, the product of the gene mutated in the human genetic disorder ataxia telangiectasia (A-T). UV-induced RPA-p34 hyperphosphorylation was not observed in A-T cells, but this response was restored by ATM expression. Furthermore, purified ATM kinase phosphorylates the p34 subunit of RPA complex in vitro at many of the same sites that are phosphorylated in vivo after UV radiation. Induction of this DNA damage response was also dependent on DNA replication; inhibition of DNA replication by aphidicolin prevented induction of RPA-p34 hyperphosphorylation by UV radiation. We postulate that this pathway is triggered by the accumulation of aberrant DNA replication intermediates, resulting from DNA replication fork blockage by UV photoproducts. Further, we suggest that RPA-p34 is hyperphosphorylated as a participant in the recombinational postreplication repair of these replication products. Successful resolution of these replication intermediates reduces the accumulation of chromosomal aberrations that would otherwise occur as a consequence of UV radiation.

INTRODUCTION

Unrepaired DNA damage can lead to mutagenesis and carcinogenesis. Exposure to DNA-damaging agents triggers signal transduction pathways that are thought to play a role in activating repair and recovery processes (Fornace, 1992; Lane, 1992; Fuks *et al.*, 1993; Herrlich and Rahmsdorf, 1994). In mammalian cells, the activation of some of these pathways appears to be initiated at the cell membrane, whereas

others appear to be triggered by the DNA damage itself (Karin and Herrlich, 1989; Karin and Hunter, 1995; Smith *et al.*, 1999b). Responses to ionizing radiation (IR) appear to be initiated by the DNA damage itself, leading to activation of serine/threonine kinases (e.g., DNA-PK and ATM) and resulting in the phosphorylation of key regulatory proteins such as p53 and CHK1 (Sanchez *et al.*, 1997; Smith *et al.*, 1999b). ATM-dependent phosphorylation events have been shown to be required for normal DNA damage-induced checkpoint control (Lavin and Khanna, 1999). The DNA-binding protein replication protein A (RPA), which plays an essential role in DNA replication, recombination, and repair (Wold, 1997; Iftode *et al.*, 1999) is also a substrate for these kinases (Zernik-Kobak *et al.*, 1997; Gately *et al.*, 1998; Chan *et al.*, 2000). RPA becomes phosphorylated as a delayed response to UV radiation (Carty *et al.*, 1994). However, the signal transduction pathway involved is thought to differ

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from that induced by IR because IR-induced phosphorylation of RPA was shown to be ATM dependent, whereas UV-induced phosphorylation of RPA appeared to occur normally in ataxia telangiectasia (A-T) cells (defective in ATM; Liu and Weaver, 1993). Because of the importance of RPA in processes required for maintaining genomic stability, such as DNA repair and recombination, we were interested in understanding more about the pathway that results in UV-induced RPA phosphorylation.

RPA is a heterotrimeric, single-stranded DNA-binding protein (subunits p70, p34, and p14) that functions in DNA replication, nucleotide excision repair, DNA recombination, double-strand break (DSB) repair, and transcriptional regulation (Wold, 1997; Iftode *et al.*, 1999). Presumably, RPA participates in these diverse functions through its strong affinity for ssDNA and its ability to interact with numerous DNA replication proteins, including DNA polymerase α and proliferating cell nuclear antigen (PCNA); nucleotide excision repair proteins XPA, XPF, and XPG; DNA recombination and DSB repair proteins Rad51, Rad52, and DNA-PK; and transcription factors such as p53 (Dutta *et al.*, 1993; He *et al.*, 1995; Matsuda *et al.*, 1995; Braun *et al.*, 1997; Loor *et al.*, 1997; Miller *et al.*, 1997; Golub *et al.*, 1998; New *et al.*, 1998; Stigger *et al.*, 1998; Shao *et al.*, 1999). The p34 subunit of RPA becomes phosphorylated during the normal cell cycle (Din *et al.*, 1990). Cell cycle-dependent phosphorylation of RPA-p34 begins at the onset of S phase and continues into mitosis, and dephosphorylation occurs in the latter part of mitosis, suggesting a physiological role for RPA-34 phosphorylation in cell cycle regulation (Din *et al.*, 1990; Fotedar and Roberts, 1992). These cell cycle-dependent phosphorylation events occur primarily at Ser-23 and Ser-29 (Dutta and Stillman, 1992), which are consensus sites for Cdc2 cyclin-dependent kinase (Pan *et al.*, 1994; Niu *et al.*, 1997). These phosphorylated forms of RPA-p34 can be observed on immunoblots of polyacrylamide gels as more slowly migrating forms (Din *et al.*, 1990; Carty *et al.*, 1994).

RPA-p34 is hyperphosphorylated in response to DNA damage, converting a significant amount of the protein to its slowest migrating form (Carty *et al.*, 1994; Zernik-Kobak *et al.*, 1997; Shao *et al.*, 1999). The kinetics of RPA-p34 hyperphosphorylation differ depending on the DNA-damaging agent. With low dose IR (2–10 Gy), hyperphosphorylation first appears at ~1–2 h and peaks at 3–4 h after treatment (Liu and Weaver, 1993; Cheng *et al.*, 1996); in contrast, with low dose UVC radiation (10–30 J/m² UV) the hyperphosphorylated form does not begin to appear until 2–4 h after treatment and reaches a maximum at around 8–10 h (Carty *et al.*, 1994). These results suggest that either the inducing signal or the signal transduction pathway leading to RPA-p34 hyperphosphorylation differs, depending on the genotoxic agent. We have shown that UV-induced hyperphosphorylation results in the phosphorylation of at least six serines and one threonine within the N-terminus of RPA-p34 (Zernik-Kobak *et al.*, 1997). All of these sites within RPA-p34 can be phosphorylated in vitro when the RPA complex (p70, p34, and p14) is incubated with purified DNA-PK and double-stranded DNA (Zernik-Kobak *et al.*, 1997). However, it is not clear what cellular enzymes are responsible for UV-induced hyperphosphorylation of RPA in vivo.

To investigate the role of the ATM kinase, we examined the capacity of A-T cells to carry out UV-induced RPA-p34 hyperphosphorylation. Here we demonstrate that A-T cells are defective in UV-induced hyperphosphorylation of RPA. When normal cells were irradiated with 10 J/m² UVC, maximal RPA hyperphosphorylation was observed at 8–12 h after exposure. In A-T cells, we did not observe the most highly phosphorylated form of RPA for 24 h after UV exposure. In addition, we used recently developed inducible cell lines (Zhang *et al.*, 1997, 1998) to show that expression of recombinant ATM protein in A-T cells corrected defective UV-induced hyperphosphorylation of RPA-p34. Furthermore, normal cells expressing antisense ATM cDNA became defective in UV-induced hyperphosphorylation of RPA-p34. A direct role for ATM in RPA-p34 hyperphosphorylation in vivo was suggested by the demonstration that purified ATM phosphorylated RPA-p34 in the purified three-subunit RPA complex in vitro on many of the same sites as are phosphorylated in vivo after UV radiation.

The time delay in UV radiation-induced RPA-p34 hyperphosphorylation suggests that this pathway is not induced directly by UV-induced photoproducts. Furthermore, because hyperphosphorylation occurs normally in nucleotide excision repair (NER)- deficient xeroderma pigmentosum group A (XPA) cells, the inducing signal does not appear to be an NER intermediate. Studies with synchronized cells suggest instead that replication of UV-damaged templates is required for generation of the inducing signal. On the basis of these observations, we postulate that the regions of single-stranded DNA and/or DNA strand breaks that are known to occur at replication forks blocked by UV-induced DNA template damage serve as the inducing signal(s) for ATM activation and RPA hyperphosphorylation.

MATERIALS AND METHODS

Cell Culture

The human skin-derived SV40-transformed fibroblast GM00637G (normal), AT5BIVA (A-T homozygous), and GM04429 (XP12BE; XPA) cell lines were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Coriell Institute for Medical Research, Camden, NJ). The SV40-transformed LM217 (normal) and AT3BISV (A-T homozygous) cell lines were generous gifts of Dr. Leon Kapp at the University of California at San Francisco. MO59K and MO59J (DNA-PK$_{cs}$ deficient) glioblastoma cells were generous gifts of Dr. Joan Allalunis-Turner (Cross Cancer Institute, Edmonton, Alberta, Canada). Recently, the two "normal" cell lines (LM217 and GM00637) were shown to be heterozygous for mutations in *ATM* (Wright *et al.*, 1996). We have detected ATM protein by Western immunoblotting in these two normal lines but not in the two A-T lines used here (our unpublished results). AT1ABR is an Epstein Barr virus (EBV)-transformed A-T lymphoblastoid human cell line that contains a homozygous in-frame 9-bp deletion (codons 2546–2548) located upstream of the PI-3 kinase domain (codons 2855–2875), which causes a lack of kinase activity (Savitsky *et al.*, 1995). These cells have been transfected with the ATM cDNA expression vector, pMAT1 with a metallothionein II promoter (Zhang *et al.*, 1997), allowing CdCl$_2$-inducible expression of ATM. C3ABR is an EBV-transformed normal lymphoblastoid cell line. These cells were transfected with a CdCl$_2$-inducible antisense ATM cDNA expression vector, pMAT2 (Zhang *et al.*, 1998).

Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO$_2$. Normal and A-T fibroblast cells were

grown in minimal essential medium (MEM) supplemented with antibiotics, 15–20% fetal bovine serum (FBS), 2% essential amino acids, 1% nonessential amino acids, and 1% vitamins. MEM and all supplements were obtained from Life Technologies (Gaithersburg, MD). Human HeLa and XP12BE cells were grown in Dulbecco's minimal essential medium (DMEM), containing 10% FBS and antibiotics, and MO59J and MO59K cells were grown in DMEM/F12 supplemented with 10% FBS and antibiotics at 37°C and 5% CO_2. Lymphoblasts were maintained in RPMI 1640 medium with 10% FBS and hygromycin B (0.2 mg/ml). Cells were maintained by subculturing at a ratio of 1:3, every 3 to 4 days, for at least 2 passages (~24 h doubling time) before plating for experimentation. Cells were not maintained in culture longer than 15–20 passages. Induction of the ATM cDNA expression vector in pMAT1-transfected AT1ABR cells and the antisense ATM expression vector in pMAT2-transfected C3ABR cells was achieved by treatment with 5 μM $CdCl_2$ for 8 h. After either $CdCl_2$ induction or mock induction, the cells were washed twice with Hanks' buffered saline solution (HBBS), and the growth medium was replaced with fresh medium. After 8 h of $CdCl_2$ induction the cells were treated with UVC radiation as described below.

Radiation Exposure

UVC radiation was delivered using a low-pressure mercury lamp (Mineralight lamp, model UVG-11; UVP, Inc., San Gabriel, CA) with maximal output at 254 nm. Before UV radiation exposure, medium was removed from the cells, and cells were washed twice in HBSS or phosphate-buffered saline (PBS; 58 mM Na_2HPO_4, 17 mM $NaHPO_4$, 68 mM NaCl, 3 mM KCl). Cells were irradiated in buffer, which was then removed, and the growth medium previously removed from the cells (held at 37° during irradiation) was returned. The cell lines AT5BIVA, GM00637, LM217, MO59J, and MO59K all had similar clonigenic survival after UVC radiation; the XP12BE line had the expected increased sensitivity to UVC-induced cell killing.

Cell Synchronization

To obtain cells primarily in S phase, asynchronous cells were treated with 6 μM aphidicolin (Sigma-Aldrich, St. Louis, MO) for 16–20 h. The medium containing aphidicolin was removed, and cells were washed twice in serum-free medium and then incubated in serum-containing medium for an additional 2–4 h. The efficacy of the aphidicolin block and release protocol in LM217 and AT5BIVA cells was measured by labeling cells with bromodeoxyuridine (BrdU). Cells were fixed onto microscope slides, and BrdU uptake was detected with FITC-labeled anti-BrdU antibody and compared with propidium iodide (PI) staining by fluorescence microscopy. PI-stained nuclei were counted, and those that also stained positive for FITC were counted as positive for DNA synthesis. After 20 h of incubation with aphidicolin, <5% of both LM217 and AT5BIVA cells were labeled with anti-BrdU; 4 h after release from the aphidicolin block, the percentage of cells labeled with anti-BrdU in both cell lines had increased to 55–60%. To obtain cells synchronized in G_1/S phase, HeLa cells were treated with 0.3 μM nocodazole for 16 h. Mitotic cells were collected by shaking the cells off the dish and pelleting them. Mitotic cells were released from nocodazole treatment for 7.5 h in fresh medium. The efficacy of the nocodazole block and release protocol was measured by double labeling cells with ^{3}H- and ^{14}C-thymidine as described below.

DNA Synthesis Assay

Thymidine incorporation was assayed essentially as described previously (Shao et al., 1997). Briefly, cells were prelabeled with 0.01 μCi/ml ^{14}C-thymidine (NEN Life Science Products, Inc., Boston, MA) for 24 h at 37°C. After incubation in fresh medium for 1 h, cells were treated with aphidicolin as above. The rate of DNA synthesis

after release from aphidicolin treatment was measured by ^{3}H-thymidine incorporation during a 30 min pulse with 10 μCi/ml ^{3}H-thymidine (NEN Life Science Products, Inc., Boston, MA). After labeling with ^{14}C- and ^{3}H-thymidine, cells were washed with PBS twice and lysed with 500 μl of 0.2 M NaOH. Triplicate samples (150 μl each) were collected onto squares of Whatman No. 3 filters (Whatman International, Maidstone, England) and rinsed with 5% trichloroacetic acid and 100% ethanol. The radioactivity of each sample was counted by dual-label liquid scintillation, and the ratio of ^{3}H/^{14}C reflected the DNA synthesis activity.

Immunoblotting

For Western immunoblots, cells were lysed in 2 ml PBSTDS (58 mM Na_2HPO_4, 17 mM $NaHPO_4$, 68 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) that included 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 0.1 mM PMSF, 5 mM NaF, and 1 mM Na vanadate. The insoluble fraction was pelleted by centrifugation at 3600 × g for 15 min at 4°C, and the supernatant was removed and stored at −70°C until use. Total protein concentration of cell lysates was determined by using the Coomassie Plus Protein assay (Pierce, Rockford, IL). Samples (50 μg total protein for RPA detection and 100 μg total protein for ATM and DNA-PK detection) were solubilized in Laemmli sample loading buffer, boiled at 100°C for 5 min, and then separated on 12% (for RPA) or 6% (for ATM and DNA-PK) denaturing SDS polyacrylamide gels (monomer to cross-linker ratio, 37.5:1) using the Laemmli buffer system (Laemmli, 1970). Proteins were transferred to Immobilon-P polyvinyl-divinyl fluoride (PVDF) transfer membranes (Millipore Corp., Bedford, MA) using a semidry apparatus (Bio-Rad Laboratories, Hercules, CA) at a maximum of 150 mA and 20 V for 1.5–2 h. The nontransferred portion of the gel was stained with 0.15% Coomassie blue to ensure equal loading of the protein samples. The membranes were blocked for 0.5–1 h with TTBS (100 mM Tris-HCl [pH 7.5], 0.9% NaCl, 0.3% Tween-20) containing 5% powdered milk and then probed with anti-ATM (1:2500; Novus Biologicals, Littleton, CO or Ab-3, Oncogene Science), anti-DNA-PK (1 μg/ml Ab-1; Oncogene Science, Cambridge, MA), anti-ATR (2 μg/ml Ab-1; Oncogene Science), or anti-RPA-p34 (2 μg/ml Ab-3, Oncogene Science; or a 1:1500 dilution of monoclonal antibody 34A [Kenny et al., 1990]) for 1–2 h. After washing four times with TTBS, the membranes were incubated with horseradish peroxidase–linked secondary antibody; sheep anti-mouse secondary antibody was used for RPA (1:2000), and donkey anti-rabbit secondary antibody was used for ATM and DNA-PK (1:1500 and 1:3000, respectively; Amersham Life Science Inc., Arlington Heights, IL). Membranes were washed three times in TTBS, and the proteins were visualized using the ECL chemoluminescent method (Amersham Life Science Inc.).

Nuclear Extract Preparation

Nuclear extracts were prepared as previously described (Andrews and Faller, 1991) with slight modifications, using hypotonic lysis followed by high salt extraction of nuclei. Briefly, frozen cell pellets were resuspended in 400-1000 μl of buffer A (10 mM HEPES-KOH [pH 7.9] at 4°C, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol [DTT], 0.5 mM PMSF, 20 mM β-glycerophosphate, 10 mM p-nitrophenylphosphate, 0.1 mg/ml pepstatin, and aprotinin). Cells were allowed to swell for 10 min, vortexed for 10 s, and centrifuged for 10 s at 23,000 × g. The pellet was resuspended in twice its volume of buffer C (10 mM HEPES-KOH [pH 7.9] at 4°C, 5% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 20 mM β-glycerophosphate, 10 mM p-nitrophenylphosphate) and incubated on ice for 20 min. The nuclear protein fraction was isolated by centrifugation for 10 min at 23,000 × g and collected as the supernatant fraction. Total protein concentration of nuclear extracts was determined by using the Coomassie Plus Protein assay (Pierce)

Purification of RPA

The RPA protein was expressed and purified from *Escherichia coli* BL21 (DE3) cells transformed with p11d-tRPA vector (a gift from Dr. Marc Wold, University of Iowa, Iowa City, IA) as described previously (Henricksen *et al.*, 1994). The p11d-tRPA vector coexpresses all three RPA subunits (RPA-p70, RPA-p34, and RPA-p14).

Purification of ATM

ATM was purified according to recently published methods (Smith *et al.*, 1999a) with minor modifications. Briefly, HeLa nuclear extracts were prepared according to Dignam *et al.* (1983) from 20 liter of HeLa S3 cells (Cell Culture Center, Minneapolis, MN) and equilibrated in buffer D (22 mM HEPES/KOH [pH 7.6], 20% glycerol, 2 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 1 mM Na metabisulfite) containing 50 mM KCl. Nuclear extract (16 ml) was loaded onto a Q-Sepharose column (20 ml, 1.6 × 10 cm; Amersham Pharmacia Biotech, Piscataway, NJ) that was equilibrated in 50 mM KCl buffer D and washed with 2.5 column volumes (50 ml) of buffer D containing 100 mM KCl. The column was eluted with a 100-ml continuous salt gradient of 50–500 mM KCl in buffer D, and 2-ml fractions were collected. The Q-Sepharose column fractions containing ATM as detected by immunoblotting were dialyzed against 2 liters of 50 mM KCl buffer D overnight. The dialysate was fractionated by size exclusion chromatography on a gel filtration column (1.6 × 100 cm with Sephacryl SR-400 HR, 10,000–2000,000; Sigma-Aldrich) equilibrated with buffer A (50 mM Tris-HCl [pH 7.5], and 100 mM KCl), collecting 1-ml fractions. Fractions containing ATM eluting in the 350- to 450-kDa range were identified by immunoblotting, pooled (16 ml total), and dialyzed overnight against 50 mM KCl buffer D. The pooled fractions were loaded onto a heparin agarose column (5 ml, 1.6 × 2.5 cm; Amersham Pharmacia Biotech) equilibrated with 50 mM KCl buffer D and washed after sample application with eight column volumes of buffer D containing 100 mM KCl. The column was eluted with a 50 ml KCl continuous gradient from 50 to 500 KCl, and 1-ml fractions eluting at 230–240 mM KCl were identified, pooled, and dialyzed against 2 liters of 50 mM KCl buffer D. The pooled ATM fractions were incubated with 150 μg of biotinylated 50-bp dsDNA conjugated to streptavidin-coated iron oxide beads (Dynal, Lake Success, NY) on a rotator at 4°C for 1 h. The ATM-DNA complexes were collected using a magnetic stand (Dynal) and washed five times with 0.5 ml of 50 mM KCl buffer D. ATM protein was eluted with 250 mM KCl buffer D (200 μl).

Protein Kinase Assays

Kinase reactions were performed by incubating purified ATM or 10 U of DNA-PK$_{cs}$/Ku (Promega, Madison, WI) or 20 U of Cdc2^{P34}/Cyclin B (New England Biolabs, Beverly, MA) at 37°C for 30 min. in 30 μl of kinase buffer (20 mM HEPES [pH 7.4], 10 mM $MgCl_2$, 100 μM ATP, 2 mM DTT, 0.2 μg of single-stranded φX174 virion DNA (ssDNA), 10 μCi [γ-^{32}P]ATP, and 0.5 μg of purified RPA). The kinase reaction was stopped by the addition of 1× Laemmli sample loading buffer. Proteins were separated on 12% denaturing SDS-PAGE gels and exposed to film.

Two-dimensional Phosphopeptide Mapping

Purified recombinant RPA complex was phosphorylated by either purified ATM or 10 U of DNA-PK for 30 min. Hyperphosphorylated RPA-p34 was separated by SDS-PAGE and transferred to a PVDF membrane. The hyperphosphorylated form was detected by phosphorimager analysis and excised from the membrane. The PVDF membrane pieces containing phosphorylated RPA-p34 were blocked with polyvinylpyrrolidone 360 (0.5% in 100 mM acetic acid) and then subjected to chymotrypsin/trypsin digestion (2 × 10 μg of chymotrypsin for 2 h at 37°C; Boehringer Mannheim, Indianapolis, IN; Luo *et al.*, 1990). After digestion, the peptides were oxidized with performic acid for 1 h at 25°C. Phosphorylated RPA-p34 chymotryptic/tryptic peptides were separated on TLC plates by electrophoresis in pH 1.9 buffer in the first dimension (anode on the left, cathode on the right) by using a model HRH gel electrophoresis apparatus (International Biotechnologies, Inc., New Haven, CT) and ascending chromatography in isobutyric acid buffer (62.5% isobutyric acid, 4.8% pyridine, 2.9% glacial acetic acid, 1.9% *n*-butanol) in the second dimension (Luo *et al.*, 1990). The phosphorylated peptides were visualized by phosphorimager analysis (Storm model 860 phosphorimager; Molecular Dynamics, Sunnyvale, CA).

RESULTS

RPA-p34 Hyperphosphorylation in Asynchronous Cell Cultures

We showed previously that hyperphosphorylation of the p34 subunit of RPA occurs 4–8 h after exposure of HeLa cells to UVC radiation (10–30 J/m²; Carty *et al.*, 1994). Because phosphorylated forms of RPA-p34 migrate slower on SDS-PAGE than do unphosphorylated forms (Carty *et al.*, 1994; Brush *et al.*, 1996), changes in phosphorylation can be measured by Western immunoblotting of cell lysates. To determine whether UV-induced hyperphosphorylation of RPA-p34 occurs normally in A-T cells, we compared the gel migration pattern of RPA-p34 from SV40-transformed skin fibroblast cell lines from normal individuals (LM217 and GM00637) and A-T patients (AT5BIVA and AT3BISV). Rapidly growing subconfluent monolayer cultures were either irradiated with 10 J/m² UVC or mock-irradiated. At various times after irradiation, whole cell lysates or nuclear extracts were prepared, and proteins were separated on SDS-PAGE, blotted to membranes, and probed with antibody against RPA-p34. In Figure 1A, mock-irradiated LM217 cells show three major cell cycle–dependent forms of RPA-p34 (bands 1–3), and the pattern of forms did not change during a 32-h time course. Lysates prepared from LM217 cells 4 h after UV exposure (10 J/m²) contained at least two additional slower migrating forms of RPA-p34 (bands 4 and 5). The intensity of the slowest migrating form (band 5, the hyperphosphorylated form) increased to a maximum at 12 h postexposure. Hyperphosphorylation of RPA-p34 was also seen in a time- (our unpublished results) and dose-dependent manner in another normal cell line, GM00637, that was exposed to 10 J/m² UVC (Figure 1C, lanes 1 and 2). The general pattern of UV-induced phosphorylation of RPA-p34 that we observed was comparable among HeLa (Carty *et al.*, 1994), LM217, and GM00637 cells.

Figure 1B shows that the three faster-migrating forms of RPA-p34 seen in LM217 cells are also observed in mock-irradiated A-T cells, although form 3 appears to be less intense than in the normal cells. However, after exposure of A-T cells to 10 J/m² UVC, there was very little change in the banding pattern of RPA-p34. Only a very faint band was observed at the position of the hyperphosphorylated form (band 5). A similar result (Figure 1C) was obtained from the analysis of UVC-induced RPA-p34 hyperphosphorylation in nuclear extracts from another normal cell line (GM00637; lanes 1 and 2) and another A-T cell line (AT3BISV; lanes 5 and 6). These data suggest that UV-induced hyperphosphorylation of RPA depends on expression of ATM.
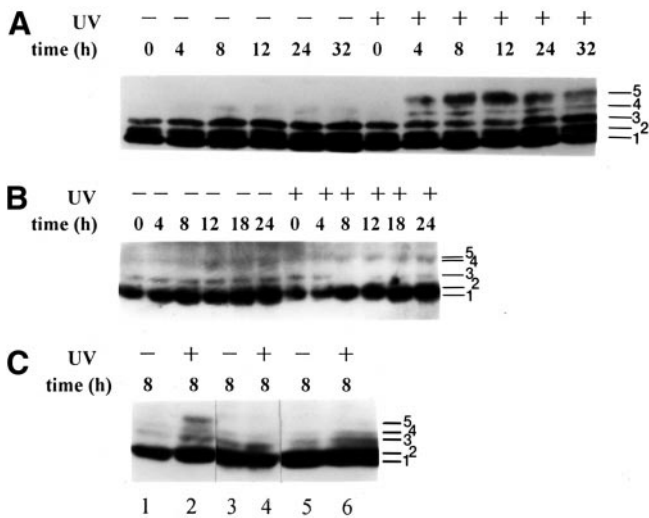
Figure 1. RPA-p34 phosphorylation in asynchronous normal and A-T cells exposed to 10 J/m² UVC. Cell lysates (A and B) or nuclear extracts (C) were prepared from asynchronously growing cells at indicated times after 10 J/m² UV radiation or mock exposure. The p34 subunit of RPA was detected by Western immunoblotting with monoclonal antibody 34A. Phosphorylation causes a slower migration on the gel; at least five different forms of the p34 subunit of RPA can be visualized. (A) RPA-p34 in LM217 cells; (B) RPA-p34 in AT5BIVA cells; (C) RPA-p34 in nuclear lysates of GM00637 (lanes 1 and 2), AT5BIVA (lanes 3 and 4), and AT3BISV (lanes 5 and 6) cells. Band 1 is unphosphorylated RPA-p34; bands 2 and 3 are cell cycle–dependent phosphorylated forms; and band 5 is the DNA damage–induced hyperphosphorylated form.

UV-induced RPA Phosphorylation in Cells Expressing Inducible Recombinant ATM Protein or ATM cDNA Antisense Transcript

In order to confirm that the difference in UV-induced hyperphosphorylation of RPA-p34 observed in A-T cells versus normal cells is due to the presence or absence of ATM, we used A-T cells expressing recombinant ATM protein or normal cells expressing ATM antisense cDNA both under control of the metallothionein promoter and inducible with $CdCl_2$. We used the A-T lymphoblastoid cell line AT1ABR, and the normal cell line C3ABR transfected with either an inducible full-length ATM cDNA or an antisense ATM cDNA, respectively (Zhang *et al.*, 1997, 1998). We confirmed by Western immunoblotting that expression of ATM was induced in the A-T cells and repressed in the normal cells by incubation in the presence of $CdCl_2$. The AT1ABR cell line contains a homozygous 9-bp in-frame deletion starting at codon 2546 of the open reading frame (Zhang *et al.*, 1997). This enables AT1ABR cells to produce a near full-length but nonfunctional protein and is consistent with our detection by immunoblotting of endogenous ATM protein in uninduced AT1ABR cells (Figure 2A, top left lane 1). As shown in Figure 2A, top left lane 2, the amount of ATM protein increased in AT1ABR cells after $CdCl_2$ treatment. The induction of antisense ATM cDNA by $CdCl_2$ in transfected C3ABR cells reduced the level of ATM protein detected at 8 h postinduction (Figure 2A, top right lane 4). The expres-
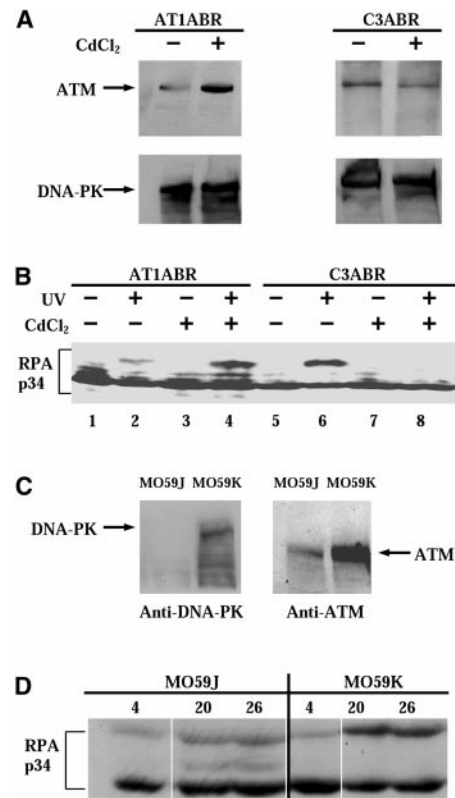

Figure 2. (A) Expression of ATM and DNA-PK proteins in pMAT1 stably transfected A-T cells (AT1ABR) and pMAT2 stably transfected normal C3ABR cells. Cells were treated with 5 μM $CdCl_2$ for 8 h. Whole-cell lysates (100 μg) were analyzed by SDS-PAGE and immunoblotted with anti-ATM (Ab-3; Oncogene Science) antibodies (top panels). AT1ABR is the parental A-T cell line containing a homozygous 9-bp in-frame deletion and is capable of producing near full-length but non-functional protein. Upon induction of the transfected line, a functional full-length recombinant ATM protein is produced. C3ABR is a control lymphoblastoid cell line expressing normal ATM. Induction of the transfected line reduces ATM expression. Membranes were reblotted with anti-DNA-PK$_{cs}$ antibody (Ab-1; Oncogene Science; bottom panels). (B) Phosphorylation of RPA-p34 after exposure of cells to UVC radiation. AT1ABR cells, transfected with a $CdCl_2$-inducible ATM cDNA expression vector pMAT1, and C3ABR cells, transfected with an inducible antisense ATM cDNA expression vector pMAT2, were treated with 5 μM $CdCl_2$ for 8 h (+) or mock-treated (−). After 8 h of $CdCl_2$ treatment, cells were UVC irradiated (30 J/m²) or mock-irradiated. Eight hours after UV treatment, whole-cell lysates were prepared and RPA-p34 was examined by gel electrophoresis, followed by immunoblotting with anti-RPA-p34 (Ab-3; Oncogene Science) antibody. (C) DNA-PK$_{cs}$ (left) and ATM (right) expression in MO59J and MO59K cells detected in whole-cell lysates by immunoblotting. (D) MO59J and MO59K cells were irradiated with 30 J/m² UV, and whole-cell lysates were prepared at the indicated times after UV irradiation. RPA-p34 hyperphosphorylation was detected by immunoblotting using anti-RPA-p34 (Ab-3; Oncogene Science) antibody. To ensure equal loading and transfer of the protein samples, the gels and the PVDF membranes were stained with Coomassie blue and colloidal gold solution, respectively.

sion of DNA-PK remained constant after $CdCl_2$ treatment in both A-T cells and normal cells (Figure 2A, bottom panels).

First, we examined whether expression of ATM in A-T cells could affect UV-induced hyperphosphorylation of

RPA-p34. Cells were grown in the presence or absence of $CdCl_2$, whole cell lysates were prepared 8 h after UVC (30 J/m^2) treatment, and lysate proteins were subjected to Western blot analysis using anti-RPA-p34 antibody. After UV irradiation, a weak band was detectable at the position of the hyperphosphorylated form of RPA-p34 in AT1ABR cells in the absence of expression of recombinant ATM protein (Figure 2B, lane 2). However, after induction of expression of full-length recombinant ATM protein in AT1ABR cells, the UV-induced hyperphosphorylation of RPA increased substantially (Figure 2B, lane 4), indicating that the expression of full-length ATM protein increased RPA-p34 phosphorylation. As expected, the mock-irradiated AT1ABR cells did not exhibit a hyperphosphorylated form regardless of $CdCl_2$ induction, although cell cycle–dependent forms of phosphorylated RPA-p34, having intermediate gel mobility, could be detected in these cells (Figure 2B, lanes 1 and 3).

We next investigated whether decreased expression of ATM protein in normal cells could affect RPA-p34 hyperphosphorylation. Cell lysates prepared from UV-irradiated (30 J/m^2), uninduced C3ABR cells showed hyperphosphorylation of RPA, as demonstrated by the presence of the slow-migrating form of RPA-p34 (Figure 2B, lane 6). This band was not observed in mock-irradiated C3ABR cells (Figure 2B, lane 5). After induction of expression of ATM cDNA antisense transcripts in the transfected C3ABR cells, hyperphosphorylation of RPA-p34 was not detected at 8 h after UV irradiation (Figure 2B, lane 8) or after mock irradiation (Figure 2B, lane 7). These data demonstrate that UV-induced hyperphosphorylation of RPA-p34 is dependent on ATM expression.

RPA-p34 Hyperphosphorylation in a DNA-PK$_{cs}$–deficient Cell Line

We showed previously that purified DNA-PK can hyperphosphorylate RPA-p34 in vitro (Zernik-Kobak *et al.*, 1997). To determine whether DNA-PK also participates in UV-induced RPA-p34 hyperphosphorylation in vivo, we compared RPA-p34 phosphorylation in DNA-PK$_{cs}$–deficient MO59J glioblastoma cells and DNA-PK$_{cs}$–expressing MO59K cells derived from the same tumor specimen (Lees-Miller *et al.*, 1995). Western blot analysis of cell lysates from DNA-PK$_{cs}$–deficient MO59J cells confirmed that these cells lack detectable DNA-PK$_{cs}$ (Figure 2C). However, these cells showed only low levels of ATM protein expression as well (Figure 2C), consistent with previous reports (Chan *et al.*, 1998; Gately *et al.*, 1998). In MO59K cells, we observed normal levels of both DNA-PK$_{cs}$ and ATM (Figure 2C). As shown in Figure 2D, RPA-p34 hyperphosphorylation after UV irradiation was observed in both MO59J and MO59K cells. The hyperphosphorylated band appears to be slightly less intense in the MO59J cells, suggesting that either DNA-PK participates in UV-induced hyperphosphorylation of RPA-p34 or that the reduced levels of ATM in these cells causes a reduction in RPA-p34 hyperphosphorylation. The fact that RPA-p34 hyperphosphorylation occurs at all in DNA-PK$_{cs}$–deficient cells (Fried *et al.*, 1996) suggests that in vivo, a kinase in addition to DNA-PK is involved in UV-induced RPA-p34 hyperphosphorylation.

Hyperphosphorylation of RPA-p34 In Vitro by Purified ATM

We next asked whether ATM kinase itself might be responsible for phosphorylation of RPA-p34. To address this question, we tested the capacity of purified ATM to phosphorylate the p34 subunit of purified RPA complex in vitro. We purified ATM from HeLa nuclei according to recently published procedures (Smith *et al.*, 1999a). We confirmed separation of ATM from DNA-PK at the heparin agarose chromatography step of the purification by Western immunoblotting of fractions from the column (Figure 3, A and B). Fractions eluting at 230–250 mM KCl (e.g., fractions 29–33, Figure 3, A–C) were pooled for further purification. ATM was further separated from ATR in the final DNA-binding step of the purification. Immunoblotting of the ATM eluate for the presence of DNA-PK and ATR (ATM- and RAD3-related protein) demonstrated that these proteins were absent from the conjugated ATM-DNA purified fractions (Figure 3D).

Purified RPA complex was incubated with purified ATM and $[\gamma\text{-}^{32}P]dATP$ in the presence of single-stranded DNA. DNA-PK and cyclin B/cdc2 kinase, which are known to phosphorylate RPA in vitro (Dutta and Stillman, 1992; Brush *et al.*, 1994; Henricksen *et al.*, 1996) were included as controls. The results in Figure 3E demonstrate that RPA-p34 becomes hyperphosphorylated in the presence of ATM. Furthermore, this kinase activity is sensitive to 50 nM wortmanin (our unpublished results), ruling out the ATR kinase, which is only sensitive to much higher concentrations (Sarkaria *et al.*, 1998). Although ATM is less active than DNA-PK, the shift in mobility of RPA-p34 is comparable with ATM and DNA-PK, suggesting that a similar number of sites on RPA-p34 are phosphorylated by the two kinases. In contrast, cyclin B/cdc2 kinase-mediated phosphorylation resulted primarily in a single labeled band, consistent with the known specificity of this kinase for only two sites (serines 23 and 29) on RPA-p34 (Pan *et al.*, 1994; Niu *et al.*, 1997). These results demonstrate that ATM has the capacity to phosphorylate the p34 subunit of RPA complex in vitro, suggesting that ATM could play a direct role in UV-induced hyperphosphorylation in vivo. These results are consistent with those of Chan *et al.* (2000), who demonstrated that ATM, purified from human placenta, had the capacity to phosphorylate RPA-p34.

Mapping of the Sites of Phosphorylation of RPA-p34 by ATM Kinase

We showed previously that DNA-PK phosphorylates the p34 subunit of the RPA complex in vitro at many of the same sites that are phosphorylated in vivo after UV-irradiation (Zernik-Kobak *et al.*, 1997). To determine whether ATM has similar substrate specificity to DNA-PK, we carried out two-dimensional peptide mapping of the phosphorylated RPA-p34 protein bands. The hyperphosphorylated form was separated by SDS-PAGE, transferred to PVDF membrane, excised, and digested with trypsin/chymotrypsin. The resulting peptides were separated in two dimensions and visualized by phosphorimager analysis (Figure 4). Overall, a similar pattern of phosphorylation was observed with ATM as with DNA-PK. Cochromatography of ATM- and DNA-PK-phosphorylated RPA-p34 confirmed those similarities
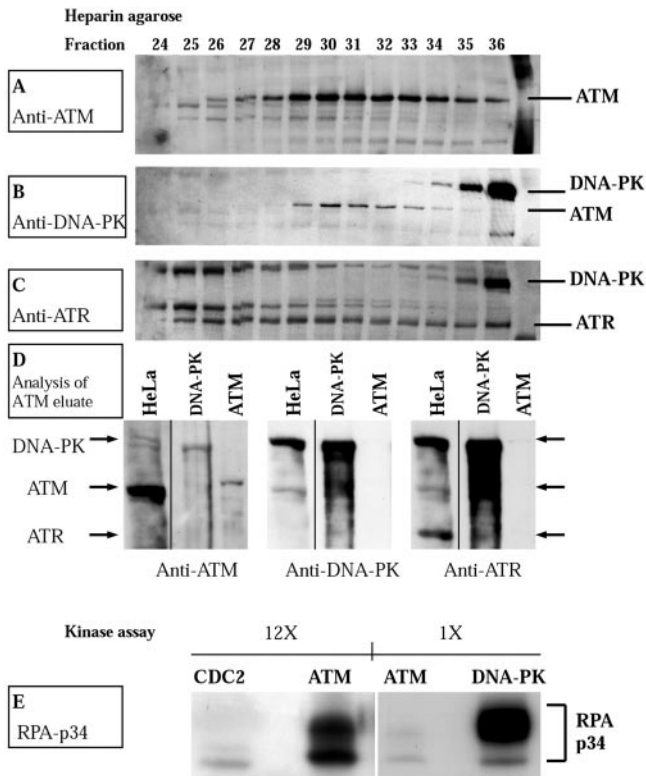
Figure 3. Purification and kinase activity of ATM. (A) Western immunoblot of fractions from heparin agarose affinity column. A 100-μl aliquot of the indicated fractions was analyzed on a 6% SDS-polyacrylamide gel, transferred to PVDF membrane, and probed with antibody to ATM (Novus Biologicals). (B) The PVDF membrane was reprobed without stripping with antibody to DNA-PK$_{cs}$ (Ab-1; Oncogene Science). (C) ATR was detected with anti-ATR (Ab-1; Oncogene Science) without stripping the membrane. (D) Western immunoblot of HeLa cell nuclear extract (70 μg), DNA-PK$_{cs}$/Ku (100 U; Promega), and purified ATM (35 μl). The samples were analyzed on a 6% SDS-polyacrylamide gel, transferred to PVDF membrane, and probed with antibody to ATM (left; Novus Biologicals). The PVDF membrane was reprobed without stripping with antibody to DNA-PK$_{cs}$ (center, Ab-1; Oncogene Science), and ATR was detected with anti-ATR (right, Ab-1; Oncogene Science). (E) Phosphorylation of RPA by Cdc2^{p34}/cyclin B (New England Biolabs) purified ATM and DNA-PK$_{cs}$/Ku (Promega). Purified recombinant RPA heterotrimer (0.5 μg) was incubated with either Cdc2^{p34}/cyclin B (20 U), purified ATM or DNA-PK$_{cs}$/Ku (10 U) in a 30-μl reaction containing 20 mM HEPES (pH 7.4), 10 mM MgCl$_2$, 100 μM ATP, 2 mM DTT, 0.2 μg of single-stranded ϕX174 virion circular DNA, and 10 μCi [γ-^{32}P]ATP. Samples were incubated for 30 min at 37°C. The kinase reaction was stopped by the addition of 1× Laemmli sample loading buffer, and samples were separated on 12% SDS-polyacrylamide gels followed by autoradiography. The designations 1× and 12× refer to the relative exposure time of the films shown.

(Figure 4, right panel). Both kinases exhibited strong phosphorylation of peptide 6 on two sites (pp6). As reported previously (Zernik-Kobak *et al.*, 1997), phosphorylation of peptide 7 on two sites (pp7) by DNA-PK could also be detected; however, with ATM it appears to be phosphorylated to a lesser extent. Although both DNA-PK and ATM

appeared to phosphorylate peptide 4 (p4), DNA-PK phosphorylated other peptides (probably peptides 2 and 3) more strongly. These results demonstrate that ATM phosphorylates RPA-p34 at multiple sites, and many of these sites appear to be the same as those phosphorylated by DNA-PK. However, there appear to be additional phosphopeptides near peptide 6 (pp6) and in the top right quadrant unique to the ATM map, suggesting some differences in the site specificities of the two kinases *in vitro*. The functional significance of these phosphorylation events remains to be determined. However, published reports suggest a role for RPA phosphorylation in DNA replication and interaction with other regulatory proteins (Iftode *et al.*, 1999).

The next series of experiments were carried out to understand more about the inducing signal for ATM-dependent, UV-induced hyperphosphorylation of RPA-p34. The fact that the time of appearance of the hyperphosphorylated form of RPA-p34 is delayed (appears at 4–8 h) after UV radiation compared with IR (appears at 1–3 h) suggests that UV-induced DNA damage (i.e., primarily pyrimidine cyclobutane dimers and 6-4 photoproducts) does not induce this pathway directly. With IR, the inducing signal is thought to be DNA strand breaks (Nelson and Kastan, 1994), although there is no direct evidence supporting this mechanism.

RPA p34 Hyperphosphorylation Does Not Require Nucleotide Excision Repair

We first tested the possibility that the signal for UV-induced hyperphosphorylation of RPA-p34 was generated as a result of nucleotide excision repair (NER) of UV photoproducts. To test whether NER was necessary for induction of RPA-p34 hyperphosphorylation after UV radiation, we used a XPA cell line, which is deficient in the recognition and excision steps of NER. UV irradiation of XPA cells led to a strong induction of hyperphosphorylated RPA-p34 (Figure 5). These results indicate that the induction of RPA-p34 hyperphosphorylation by UV irradiation does not require active NER.

RPA p34 Hyperphosphorylation Is Associated with Replication of UV-damaged DNA

Replication of UV-damaged DNA templates leads to replication fork blockage at sites of damage (Park and Cleaver, 1979; Bierne and Michel, 1994; Seigneur *et al.*, 1997). If blockage occurs on the leading strand, continued replication on the lagging strand leads to the accumulation of long stretches of single-stranded parental DNA (Wang and Smith, 1986; Kaufmann, 1989; Michel *et al.*, 1997). In addition, DNA double-strand breaks occur at blocked replication forks (Wang and Smith, 1986; Kaufmann, 1989; Michel *et al.*, 1997). If these abnormal DNA structures serve as signals for induction of RPA-p34 hyperphosphorylation, we would expect that induction would occur more intensely and more rapidly if synchronized cells were irradiated at the beginning of S phase. To test this hypothesis, cells were synchronized by nocodazole and then released for 7.5 h to place them at the G$_1$/S border (Figure 6B). Cells were then irradiated with 30 J/m^2 UV and incubated further in the presence or absence of aphidicolin (Figure 6C). In the absence of aphidicolin, hyperphosphorylation of RPA-p34 was ob-
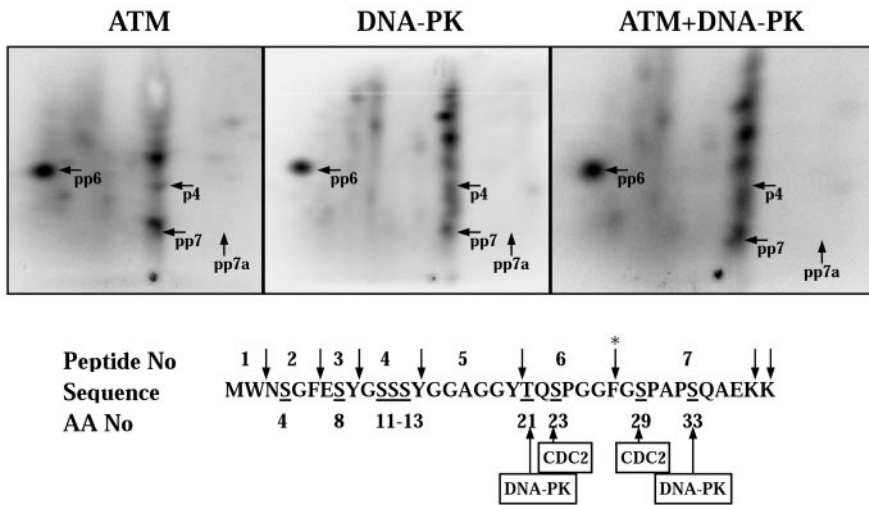
Figure 4. Chymotryptic/tryptic phosphopeptide maps of in vitro–phosphorylated recombinant RPA-p34. (Top) Five hundred nanograms of purified recombinant RPA complex was phosphorylated by either purified ATM or 10 U of DNA-PK$_{cs}$/Ku for 30 min. Hyperphosphorylated RPA-p34 was separated by SDS-PAGE and transferred to a PVDF membrane. The hyperphosphorylated form of RPA-p34 was detected by phosphorimager analysis and excised from the membrane. The hyperphosphorylated RPA-p34 was digested twice with 10 μg of chymotrypsin/trypsin and oxidized with performic acid. The digested peptides were loaded onto TLC plates and separated by electrophoresis at pH 1.9 in the first dimension, followed by ascending chromatography in the second dimension. The labeled peptides were detected by phosphorimager analysis. To verify identical and unique peptides, equal radioactivity from digests of DNA-PK$_{cs}$/Ku and ATM-phosphorylated RPA-p34 were loaded onto the same chromatography plate and subjected to two-dimensional separation. Numbered tryptic/chymotryptic peptides indicated on the maps with arrows refer to the peptide sequence number (lower panel) with a letter "p" designating a phosphorylated serine or threonine on the peptide. (bottom) The amino acid sequence of the N-terminus of RPA-p34 is shown along with the sites for cleavage by trypsin/chymotrypsin (↓), sites phosphorylated by Cdc2^{p34}/cyclin B and consensus sites for DNA-PK$_{cs}$/Ku. The asterisk denotes the cleavage site that is blocked by adjacent phosphorylated amino acids.

served by 8 h after UV irradiation. In the presence of aphidicolin, the UV-induced hyperphosphorylation RPA-p34 was absent. These data support the conclusion that UV-induced RPA-p34 hyperphosphorylation is associated with replication of UV-damaged DNA.

ATM Dependence of RPA-p34 Hyperphosphorylation in Aphidicolin-synchronized Cell Cultures

The next experiment was carried out to determine whether the hyperphosphorylation of RPA-p34 observed in aphidicolin-treated cells after UV-irradiation was ATM dependent. To test this hypothesis, we treated cells for 17 h with aphidicolin, which inhibits the elongation step of DNA replication and blocks cell cycle progression at the G$_1$/S border (Pedrali-Noy *et al.*, 1982). Cells were then released from the block by transferring them to fresh medium without aphidi-

Figure 5. Time course of RPA phosphorylation after irradiation of XP12BE cells (XP complementation group A). XPA cells were either mock-irradiated or treated with the indicated doses of UVC. At the indicated times after treatment, cell lysates were prepared and the RPA-p34 phosphorylation pattern was analyzed by gel electrophoresis, followed by immunoblotting. The arrow to the right indicates the major hyperphosphorylated form of RPA-p34.

colin. After an additional 2-h incubation, cells were irradiated with 10 J/m² UVC or mock-irradiated (Figure 7A). Cell lysates were prepared at the indicated times thereafter, and the phosphorylation state of RPA-p34 was analyzed by Western immunoblotting. We confirmed that the cells released from the aphidicolin block had a rapid increase in DNA synthetic activity, by measuring the incorporation of [³H]thymidine (Figure 7B). We also confirmed that the DNA content of cells increased by 5 h after the release of aphidicolin block (our unpublished results). Strong hyperphosphorylation of RPA p34 was observed by 4 h after UV irradiation of aphidicolin-synchronized HeLa cells (Figure 7C). Immediately after irradiation or mock irradiation, the majority of RPA-p34 in lysates prepared from LM217 or AT5BIVA cells was in faster migrating forms of the subunit (particularly in the doublet of bands 1 and 2; Figure 7D, lanes 1, 2, 5, and 6). Eight hours after mock irradiation, this pattern did not change appreciably (Figure 7D, lanes 3 and 7). At 8 h after 10 J/m² UVC exposure, however, a slow-mobility form (band 5) was apparent in lysates from LM217 cells (Figure 7D, lane 8) but not from A-T cells (Figure 7D, lane 4). These data support the observation that UV-induced hyperphosphorylation of RPA-p34 does not occur normally in A-T cells.
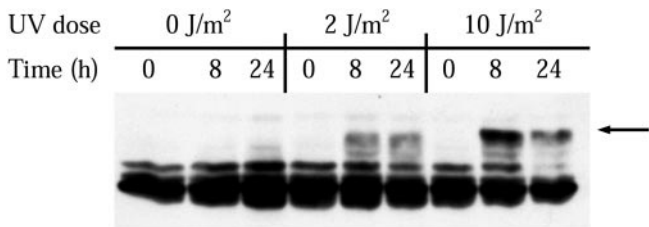
DISCUSSION

We have investigated the requirements for UV radiation–induced RPA-p34 hyperphosphorylation. Western immunoblotting revealed that the p34 subunit of the RPA protein complex was hyperphosphorylated in a time- and dose-dependent manner in normal, asynchronously growing cells after exposure to UVC (Carty *et al.*, 1994). This response was both delayed and reduced in intensity in A-T cells. We have
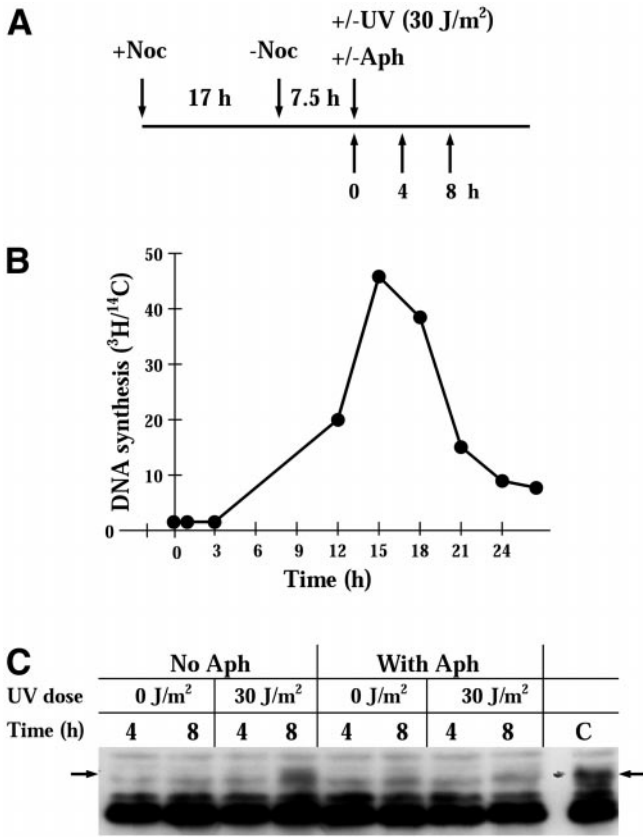
Figure 6. UVC-induced RPA-p34 hyperphosphorylation in the presence of aphidicolin. (A) Protocol for treatment. HeLa cells were treated with 0.3 μM nocodazole for 16 h. Mitotic cells were collected by shaking the cells off the dish and pelleting them. To obtain cells synchronized in G_1/S phase, mitotic cells were released from nocodazole treatment for 7.5 h in fresh medium. Cells were either mock-irradiated or irradiated with 30 J/m^2 UVC in the presence or absence of aphidicolin (6 μM). (B) The rate of DNA synthesis after release from nocodazole treatment was measured as ^{3}H-thymidine incorporation during a 30-min pulse with 10 μCi/ml ^{3}H-thymidine (NEN Life Science Products, Inc.). Cells were prelabeled with 0.01 μCi/ml ^{14}C-thymidine (NEN Life Science Products, Inc.) for 24 h at 37°C, followed by ^{3}H-thymidine pulse labeling. After labeling, cells were washed with PBS twice and lysed with 500 μl of 0.2 M NaOH per dish. The radioactivity of each sample was counted by dual-label liquid scintillation, and the ratio of ^{3}H/^{14}C reflected the DNA synthesis activity. (C) RPA-p34 phosphorylation in nocodazole-synchronized HeLa cells exposed to UVC. Cells in G_1/S phase were either mock-irradiated or irradiated with 30 J/m^2 UVC in the presence or absence of aphidicolin (6 μM). After preparation of whole-cell lysates, RPA-p34 was visualized by immunoblotting with anti-RPA-p34 antibody and ECL chemiluminescent detection.

also used recombinant ATM and antisense ATM expression vectors to demonstrate that UV-induced RPA-p34 hyperphosphorylation is dependent on the expression of ATM. Reduction of ATM levels in normal C3ABR cells, by the expression of an antisense cDNA, decreased UV-induced RPA-p34 hyperphosphorylation. Furthermore, expression of a full-length recombinant ATM protein in AT1ABR cells fully restored UV-induced RPA-p34 hyperphosphorylation
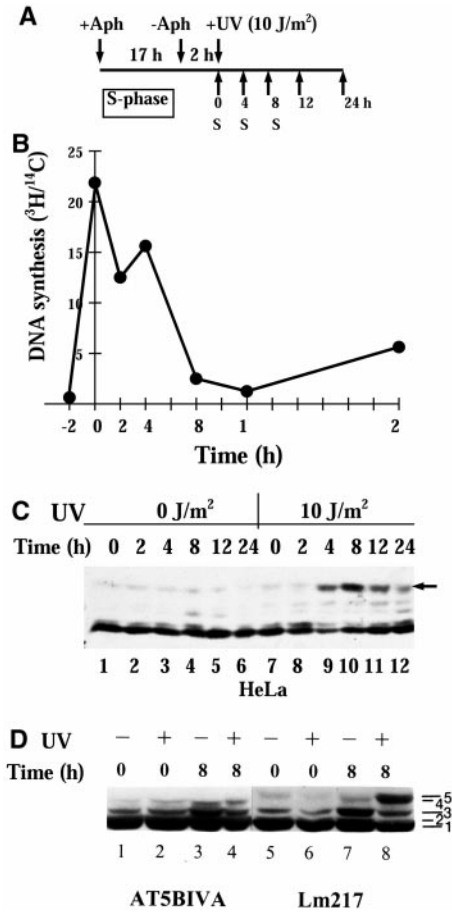

Figure 7. UV-induced RPA-p34 phosphorylation in aphidicolin-synchronized cells. (A) Protocol for treatment. Cells were treated with 6 μM of aphidicolin for 16–20 h. After the medium containing aphidicolin was removed; the cells were washed twice in serum-free medium and further incubated in serum-containing medium without aphidicolin for 2–4 h. After 2–4 h in fresh medium, the cells were mock-irradiated or irradiated with 10 J/m^2 UVC and then incubated in the same medium for the indicated times. (B) The rate of DNA synthesis after release from aphidicolin treatment was measured as ^{3}H-thymidine incorporation during a 30-min pulse with 10 μCi/ml ^{3}H-thymidine (NEN Life Science Products, Inc.). Cells were prelabeled with 0.01 μCi/ml ^{14}C-thymidine (NEN Life Science Products, Inc.) for 24 h at 37°C followed by ^{3}H-thymidine pulse labeling. After labeling, cells were washed with PBS twice and lysed with 500 μl of 0.2 M NaOH per dish. The radioactivity of each sample was counted by dual-label liquid scintillation and the ratio of ^{3}H/^{14}C reflected the DNA synthesis activity. (C) Time course of RPA-p34 phosphorylation in HeLa cells. Whole cell lysates were prepared at various times after mock irradiation or irradiation with 10 J/m^2 UVC. Proteins were separated on a 12% denaturing polyacrylamide gel. After transfer of the proteins to a PVDF membrane RPA-p34 phosphorylation pattern was analyzed by immunoblotting using an antibody specific for RPA-p34 (monoclonal antibody 34A). (D) RPA-p34 phosphorylation in aphidicolin synchronized normal Lm217 and A-T cells exposed to UVC. Cells were irradiated with 10 J/m^2 UVC or mock-irradiated 2 h after release from the aphidicolin block. Cell lysates were prepared 0 or 8 h after irradiation. The p34 subunit of RPA was analyzed by immunoblotting.

in these cells. We conclude that ATM expression is required for UV-induced RPA-p34 hyperphosphorylation.

Our conclusions appear to differ from those of Liu and Weaver (1993), who observed that A-T cells were deficient in hyperphosphorylation of RPA-p34 in response to IR but not UV radiation. With IR, hyperphosphorylation of RPA-p34 was reduced and delayed in A-T cells compared with normal cells. UV-induced hyperphosphorylation of RPA-p34 was examined only up to 3 h after irradiation (Liu and Weaver, 1993). At these early times, little difference was observed between A-T cells and normal cells; after 500 J/m² UV, strong hyperphosphorylation of RPA-p34 was observed in both A-T and normal cells; after 50 J/m² UV, hyperphosphorylation of RPA-p34 was observed in some A-T and normal cells but not others; and after 10 J/m² UV, little hyperphosphorylation was observed in either cell type. Because the ATM-dependent phosphorylation pathway we are investigating was induced at later times (4–8 h after UV irradiation) and at lower UV fluences (10–30 J/m²), our results are not, in fact, in direct conflict with those of Liu and Weaver (1993).

Our observation that purified ATM has the capacity to phosphorylate RPA-p34 in vitro suggests that the ATM kinase may participate directly in UV-induced RPA-p34 hyperphosphorylation in vivo. However, it is possible that the requirement of ATM for UV-induced RPA-p34 hyperphosphorylation is indirect and other kinases may be involved. We showed previously that DNA-PK phosphorylates in vitro many of the same sites within the N-terminus of RPA-p34 that are phosphorylated in vivo after UV-irradiation (Ser-11, Ser-12, or Ser-13 on peptide 4; Thr-21and Ser-23 on peptide 6; Ser-29 and Ser-33 on peptide 7; also probably Ser 4 on peptide 2, and Ser 8 on peptide 3; Zernik-Kobak *et al.*, 1997). However, several lines of evidence suggest that DNA-PK is not the only kinase required for RPA-p34 phosphorylation after DNA damage. For example, human DNA-PK$_{cs}$–deficient glioblastoma cells M059J hyperphosphorylated RPA-p34 after IR (Fried *et al.*, 1996). This is in agreement with our observation that RPA-p34 hyperphosphorylation occurred in UV-irradiated M059J cells, albeit to a lesser extent. Our observations agree with other reports that M059J cells have extremely low levels of ATM protein. The lower levels of ATM, rather than the lack of DNA-PK, may account for the decreased levels of hyperphosphorylated RPA-p34 observed in these cells after genotoxic insult (Chan *et al.*, 1998; Gately *et al.*, 1998). A role for ATM in RPA-p34 phosphorylation is also supported by the demonstration that ATM immunoprecipitates from MO59J cells retain in vitro kinase activity toward RPA-p34 (Gately *et al.*, 1998). Here we show that the sites of phosphorylation of RPA-p34 by ATM in vitro are similar to those of DNA-PK and therefore similar to those that occur in vivo. It is possible that both DNA-PK and ATM may participate in some way in UV-induced RPA-p34 hyperphosphorylation.

Another candidate for in vivo phosphorylation of RPA-p34 is ATR. Expression of kinase-inactive ATR protein (ATRkd) in human fibroblasts increased sensitivity to UV and IR and led to loss of cell cycle checkpoint control (Wright *et al.*, 1998). Although ATR kinase does not phosphorylate RPA-p34 in vitro (Hall-Jackson *et al.*, 1999), the functional overlap between ATM and ATR suggests that RPA-p34 may be an in vivo substrate for ATR. Although
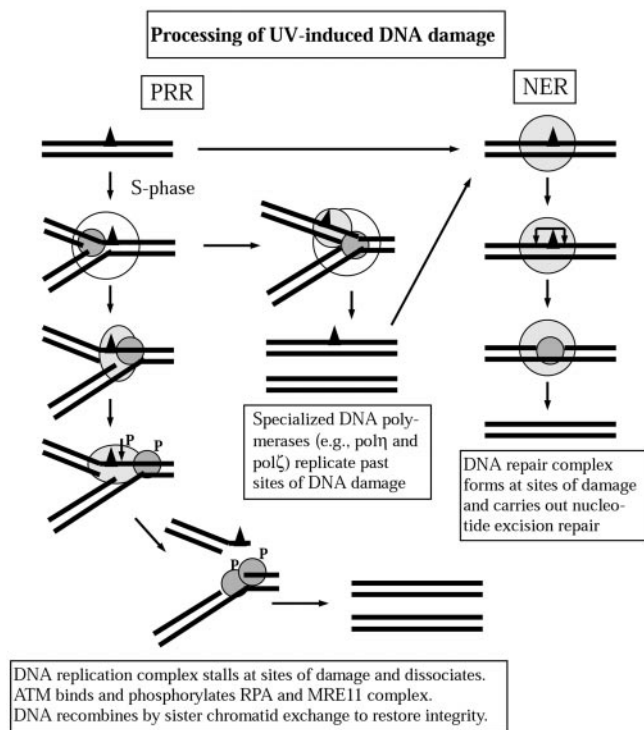

Figure 8. Model for cellular processing of UV-induced DNA damage. UV radiation induces mainly pyrimidine cyclobutane dimers and 6-4 photoproducts. The majority of these are repaired by nucleotide excision repair (NER), which involves recognition of the site of damage by XPA with the assistance of RPA and the recruitment of additional repair proteins that incise the DNA on either side of the damage, release a single-stranded oligonucleotide containing the damage and fill in the resulting gap. If cells are in S phase and DNA replication occurs before all the damage is repaired, the DNA replication fork may encounter sites of damage. One of several specialized DNA polymerases may facilitate replication past the damage. If replication becomes stalled at sites of DNA damage, mechanisms of postreplication repair (PRR) are activated. This activation appears to involve the ATM kinase, which phosphorylates RPA and NBS1. The MRE11/RAD50/NBS1 complex may participate in processing the incompletely replicated DNA, facilitating recombinational repair.

ATM has been implicated in IR-induced RPA-p34 hyperphosphorylation and ATM-immune precipitates of IR-treated cells phosphorylated RPA-p34 (Gately *et al.*, 1998), a direct role for ATM kinase in IR-induced hyperphosphorylation has also not yet been demonstrated.

The signal for IR-induced, ATM-dependent RPA-p34 hyperphosphorylation is thought to be IR-induced DNA strand breaks (Liu and Weaver, 1993), although no direct evidence for this hypothesis has been provided. UV radiation induces primarily cyclobutane pyrimidine dimers and 6-4 photoproducts. Because UV-induced RPA-p34 hyperphosphorylation only occurs several hours after UV irradiation, UV photoproducts are not likely to be the primary inducing signal. There are several different pathways by which cells process UV-induced DNA damage (Figure 8) that might generate the signal for ATM-dependent RPA-p34 hyperphosphorylation. In normal cells, most UV-induced

DNA damage is repaired by NER, which generates DNA single-strand breaks as transient intermediates (Kaufmann and Wilson, 1990). However, nucleotide excision repair does not appear to be required for induction of RPA-p34 hyperphosphorylation by UV radiation, because we showed that hyperphosphorylation was induced normally in XPA cells.

Our observations and others (Rodrigo *et al.*, 2000) that UV-induced RPA-p34 hyperphosphorylation was blocked when DNA replication was inhibited by aphidicolin argue that the inducing signal for ATM-dependent RPA-p34 hyperphosphorylation is generated during replication of UV-damaged DNA templates. Models for replication of UV damaged DNA templates and postreplication repair were first proposed in the early 1970s to explain the induction of chromosomal aberrations by UV and chemical carcinogens (Bender *et al.*, 1973, 1974); these models have been refined more recently to include new information on mechanisms of postreplication repair (Seigneur *et al.*, 1998). When DNA replication occurs while UV-photoproducts remain in the DNA, some of these lesions block the progression of the DNA replication fork (Park and Cleaver, 1979). This blockage is sometimes overcome by the action of DNA polymerases (e.g., polymerases η and ζ and the product of hREV1) that have the capacity to bypass UV photoproducts (Gibbs *et al.*, 1998; Lin *et al.*, 1999; Masutani *et al.*, 1999). However, if the blockage persists, aberrant DNA replication intermediates are produced that contain long regions of single-stranded DNA; double-strand DNA breaks also accumulate (Wang and Smith, 1986; Kaufmann, 1989; Michel *et al.*, 1997). Although the exact nature of the inducing signal remains to be elucidated, it is interesting to note that ATM-dependent RPA-p34 phosphorylation in vitro is activated by closed circular single-stranded DNA. If the double-strand breaks that accumulate after UV radiation are the inducing signal, this would suggest similarities between the signals for IR- and UV-induced ATM activation. Recently, it was shown that DNA replication was also required for induction of RPA-p34 hyperphosphorylation by adozelesin, a DNA-alkylating agent, but not by IR (Liu *et al.*, 2000). Like UV, adozelesin would be expected to cause DNA replication–blocking lesions.

The aberrant replication intermediates that accumulate after UV radiation are thought to be resolved through a recombinational mechanism between sister chromatids (Bender *et al.*, 1973, 1974; Kaufmann, 1989; Haber, 2000; Lowndes and Murguia, 2000). However, the enzymology of this process is not yet understood. Recently, Limoli *et al.* (2000) demonstrated colocalization of PCNA (involved in DNA replication) and the MRE11 complex (Mre11/Rad50/Nbs1, involved in recombinational repair) in foci 4–8 h after UV radiation of XPV human fibroblasts (deficient in DNA polymerase η). This result implicates the MRE11 complex in recognition and/or resolution of DNA replication intermediates after UV radiation. Indeed, it has been suggested that this complex performs a similar role during replication of undamaged templates (Petrini, 2000). Because NBS1 appears to be a substrate for the ATM kinase (Gatei *et al.*, 2000; Lim *et al.*, 2000), it is possible that MRE11 focus formation in response to UV damage also involves ATM (Petrini, 2000). ATM-dependent RPA-p34 hyperphosphorylation may occur in concert with phosphorylation of other members of this complex.

ATM-dependent phosphorylation events are involved in IR-induced checkpoint control. The radioresistant DNA synthesis observed in A-T cells is attributed to the loss of the S-phase checkpoint. Interestingly, mutation of the ATM kinase phosphorylation site on NBS1 also leads to loss of the IR-induced, S-phase checkpoint (Lim *et al.*, 2000). This observation suggests that the MRE11 complex may play a role in IR-induced ATM-dependent cell signaling. Whether phosphorylation of NBS1 or RPA plays a role in UV-induced checkpoint control has not yet been determined.

Presently, there is no definitive evidence for a functional role of RPA hyperphosphorylation. However, a number of lines of evidence suggest that phosphorylation may alter the activity of RPA for DNA replication, NER, and double-strand break repair (reviewed in Iftode *et al.*, 1999). The strong binding affinity of the three-subunit RPA complex for ssDNA is predominantly attributed to the RPA-p70 subunit; the 34-kDa subunit is thought to play a regulatory role (Wold, 1997; Bochkareva *et al.*, 1998; Iftode *et al.*, 1999). RPA also binds with high affinity to certain double-stranded DNA regulatory sequences (Singh and Samson, 1995; Tang *et al.*, 1996) and to double-stranded DNA carrying UV photoproducts or *cis*-platin DNA cross links (Clugston *et al.*, 1992; Burns *et al.*, 1996; Patrick and Turchi, 1998). RPA has a double-stranded DNA unwinding activity, which is altered by phosphorylation (Georgaki *et al.*, 1992; Georgaki and Hubscher, 1993). We have shown that the DNA replication capacity was reduced in extracts from UV-irradiated human cells (containing hyperphosphorylated RPA), but replication capacity could be restored by the addition of purified RPA (Carty *et al.*, 1994). A similar observation was recently made with extracts from adozelesin-treated cells (Liu *et al.*, 2000). Park *et al.* (1999) reported DNA synthesis inhibition in RPA-enriched replication extracts from UV-treated MO59K (DNA-PK$_{cs}$+) cells but not in cell extracts from MO59J (DNA-PK$_{cs}$−) cells, implying modulation of RPA by DNA-PK. However, interpretation of these data is complicated by the fact that the DNA-PK$_{cs}$–deficient glioblastoma cells (MO59J) also have reduced amounts of ATM compared with the control line (MO59K). Rodrigo *et al.* (2000) reported a temporal parallel between RPA-p34 hyperphosphorylation and DNA synthesis inhibition after UVC irradiation, and Shao *et al.* (1999) reported a similar relationship after camptothecin treatment. In addition, Henricksen *et al.* (1996) demonstrated that phosphorylation of RPA-p34 modulates DNA replication. Also, RPA phosphorylation reduces the ability of RPA to interact with DNA polymerase α-primase (Iftode *et al.*, 1999) and p53 (Abramova *et al.*, 1997). It is not clear how these in vitro observations relate to RPA function in vivo and in particular to DNA repair functions or the S-phase checkpoint.

What is the biological significance of a role for ATM in cellular responses to UV-induced DNA damage? A-T patients are acutely sensitive to ionizing radiation, but no enhanced sensitivity to UV radiation has been documented (Paterson and Smith, 1979; Sedgwick and Boder, 1991; Woods and Taylor, 1992; Taylor *et al.*, 1994). Likewise A-T cells exhibit enhanced sensitivity to killing by IR but not UV. However, there are abnormalities in the response of A-T cells to UV radiation. Both the accumulation of DNA strand breaks and the subsequent generation of chromosome aberrations are elevated in UV-irradiated A-T cells (Sasaki, 1980;

Ejima and Sasaki, 1986; Kaufmann, 1989; Kaufmann and Wilson, 1994). Kaufmann and Wilson demonstrated that the greatest numbers of chromosomal aberrations occurred when normal cells were UV irradiated at the G1/S border and that A-T fibroblasts were hypersensitive to the induction of chromosomal aberrations by UV (Kaufmann and Wilson, 1994). The fact that UV induces mainly chromatid-type chromosomal aberrations suggests that they arise after DNA replication has occurred (Kaufmann, 1989). This abnormality in response to UV in A-T cells likely reflects an abnormality in response to DNA replication-blocking lesions in general, which would include those caused by carcinogens that form bulky adducts (e.g., benzo[a]pyrene) and alkylated bases (e.g., methyl methane sulfonate). Indeed a very similar response was observed in adozelesin-treated cells (Liu *et al.*, 2000). Perhaps this defect in response to a broader range of DNA-damaging agents accounts for the appearance of a broad spectrum of cancers in adult A-T patients (Meyn, 1999). Recently ATM has been linked to other chromosome instability syndromes through its apparent interaction with the MRE11/RAD50/NBS1 complex. The *MRE11* gene has been found to be mutated in two patients with an A-T-like syndrome (Stewart *et al.*, 1999), and the *NBS1* gene is mutated in Nijmegen breakage syndrome (Gatei *et al.*, 2000; Lim *et al.*, 2000; Petrini, 2000; Wu *et al.*, 2000; Zhao *et al.*, 2000). Perhaps all of these diseases share a postreplication repair defect.

In summary, we have demonstrated that the RPA-p34 hyperphosphorylation that occurs in response to UV radiation is dependent on DNA replication and expression of the ATM kinase. We postulate that this pathway is induced in response to abnormal DNA replication intermediates that accumulate as a result of DNA replication fork blockage by UV photoproducts. Further, we suggest ATM plays a role in signaling to components of the postreplication repair apparatus, including RPA, to trigger recombinational repair and prevent the formation of chromosomal aberrations resulting from unresolved replication intermediates.

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