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DNA Replication but Not Nucleotide Excision Repair Is Required for UVC-Induced Replication Protein A Phosphorylation in Mammalian Cells

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Exposure of mammalian cells to short-wavelength light (UVC) triggers a global response which can either counteract the deleterious effect of DNA damage by enabling DNA repair or lead to apoptosis. Several stress-activated protein kinases participate in this response, making phosphorylation a strong candidate for being involved in regulating the cellular damage response. One factor that is phosphorylated in a UVC-dependent manner is the 32-kDa subunit of the single-stranded DNA-binding replication protein A (RPA32). RPA is required for major cellular processes like DNA replication, and removal of DNA damage by nucleotide excision repair (NER). In this study we examined the signal which triggers RPA32 hyperphosphorylation following UVC irradiation in human cells. Hyperphosphorylation of RPA was observed in cells from patients with either NER or transcription-coupled repair (TCR) deficiency (A, C, and G complementation groups of xeroderma pigmentosum and A and B groups of Cockayne syndrome, respectively). This exclude both NER intermediates and TCR as essential signals for RPA hyperphosphorylation. However, we have observed that UV-sensitive cells deficient in NER and TCR require lower doses of UV irradiation to induce RPA32 hyperphosphorylation than normal cells, indicating that persistent unrepaired lesions contribute to RPA phosphorylation. Finally, the results of UVC irradiation experiments on nonreplicating cells and S-phase-synchronized cells emphasize a major role for DNA replication arrest in the presence of UVC lesions in RPA UVC-induced hyperphosphorylation in mammalian cells.

Exposure of mammalian cells to short-wavelength light (UVC) triggers a global response which can either enable DNA repair or lead to apoptosis. UVC-induced reactions are broadly classified as an immediate response occurring within minutes upon irradiation and a late response detectable only after a lag following UVC exposure (for a review, see reference 5). The major UVC-induced event is transcription modulation, which in turn adapts the various cellular functions to the stress conditions. The molecular origin of the UVC-induced signal remains unclear, but molecular responses show the same two-phase temporal response. Several receptor tyrosine kinases and protein kinases at the plasma membrane are rapidly activated after UVC irradiation (5), even in enucleated cells (20). In contrast, the extent of persistent UVC photoproducts in DNA controls later events like transcription of delayed UVC-responsive genes (7, 66).

Many UVC-induced reactions rely on posttranslational modifications of preexisting proteins. Beside participating in signaling cascades initiated from the plasma membrane, protein phosphorylation is also implicated in the checkpoint pathways that control cell cycle progression following DNA damage. For example, DNA damage induces phosphorylation of p53 tumor suppressor gene product which correlates with both the accumulation of the protein and the activation of its transactivation properties toward cell cycle controlling genes (63, 64).

Hyperphosphorylation of replication protein A (RPA) is one of a number of UVC-induced phosphorylation events (14, 47). RPA is a highly conserved eukaryotic protein which is required for DNA replication, repair, and recombination (for a review, see reference 71). In human cells, it is the most abundant single-stranded DNA-binding protein. Human RPA is a stable heterotrimer of three subunits of approximately 70, 32, and 14 kDa (RPA70, RPA32, and RPA14, respectively). Although RPA binding to single-stranded DNA is mediated by the RPA70 subunit (42, 73), all three subunits are necessary for RPA functions since all the three genes encoding subunits of RPA are essential in yeast (10).

RPA was initially identified as an essential component for simian virus 40 (SV40) replication in vitro. Interactions of RPA with SV40 T antigen and the DNA polymerase α primase are necessary for initiation at the origin sequence (25, 70, 72). In addition, RPA is involved in the elongation phase by stimulating the action of several DNA polymerases (9, 24, 41, 68).

RPA is also an essential component in the nucleotide excision repair (NER) process, the major DNA repair pathway for numerous DNA lesions including UVC photoproducts (for a review, see reference 74). The reaction is carried out by the coordinated action of about 25 proteins that are involved in the two main steps: recognition/incision-excision of the lesion and DNA synthesis/ligation to restore strand continuity (60). As a DNA polymerase-interacting factor, RPA is necessary for the DNA synthesis step (18, 19). In addition, RPA is absolutely required for the dual incisions around the lesion on the damage strand (1, 32, 51), which generate a ~30-nucleotide single-stranded region corresponding to the RPA footprint on DNA (6, 44). RPA has been shown to interact with XPA, XPF-ERCC1, and XPG NER proteins in extracts from human cells (34, 45, 49, 59) and with TFIIH in yeast extracts (37).

In yeast and mammalian cells, the RPA32 subunit has been

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shown to be phosphorylated both in a cell cycle-dependent manner (21, 26) and in response to various DNA-damaging treatments (12, 14, 47, 62, 69). Phosphorylated forms were easily distinguishable by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The slowest-migrating hyperphosphorylated forms were detected only after treatment with DNA-damaging agents (14, 47) and were clearly distinguished from other forms of lower level of phosphorylation from normally cycling cells. Indeed, analysis of phosphorylated RPA32 from HeLa cells after UVC exposure identified at least seven phosphorylated sites in the slowest-migrating forms, compared with three to five in the forms from unirradiated control cells (77).

RPA32 hyperphosphorylation was reproduced in vitro during SV40 replication with human cell extracts (27). RPA32 phosphorylation in vitro was shown to occur specifically during replication initiation after RPA has bound to single-stranded DNA at the unwound origin (27). Other observations also indicate that hyperphosphorylation of RPA32 requires RPA binding to DNA (26, 35, 36). Accordingly, it has been shown that RPA binding to single-stranded DNA induces a new conformation that facilitates its phosphorylation (6, 31). Thus, damage-induced RPA phosphorylation is more likely to occur after the protein has bound to activating single-stranded DNA structures.

Here, we have examined which cellular signal triggers RPA hyperphosphorylation following UVC irradiation in human cells. In UVC-damaged cells, single-stranded DNA sites may occur as intermediates during both DNA repair and DNA replication arrest caused by the presence of UVC lesions. We have examined the roles of NER and DNA replication in RPA32 phosphorylation following UVC exposure of human fibroblasts. Hyperphosphorylation of RPA was observed in cells from patients with either NER or transcription-coupled repair (TCR) deficiency (A, C, and G complementation groups of xeroderma pigmentosum [XP-A, XP-C, and XP-G] or A and B groups of Cockayne syndrome [CS], respectively). This excludes both NER intermediates and TCR as essential signals for RPA hyperphosphorylation. However, we have observed that UV-sensitive cells deficient in NER and TCR require lower doses of UV irradiation to induce RPA32 hyperphosphorylation than normal cells, indicating that persistent unrepaired lesions are responsible for signal generation. Finally, the results of UVC irradiation experiments on nonreplicating cells and synchronized cells emphasized a major role for DNA replication arrest in the presence of UVC lesions in the route leading to RPA UVC-induced hyperphosphorylation in mammalian cells.

MATERIALS AND METHODS

Cell culture and chemicals. All of the SV40-transformed human fibroblasts used here were obtained from A. Sarasin and M. Mezzina (Centre National de la Recherche Scientifique, Villejuif, France). MRC5 was derived from a normal individual, and XP-A (XP12ROSV), XP-C (XP4PASV), and XP-G (XP3BESV) were derived from patients suffering from xeroderma pigmentosum, and CS-B (CS1AN) was from a patient suffering from Cockayne syndrome. The XP-At cell line was derived from the XP-A cell line by transfection with a recombinant retroviral vector (LXPASN) bearing the human *XPA* cDNA and conferring resistance to geneticin (76). All cell lines were grown in alpha minimal essential medium (Biomedia) supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (125 U/ml), and streptomycin (125 μg/ml). For XP-At selection, geneticin (150 μg/ml) (Gibco-BRL) was added to the medium. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

All of the above reagents were purchased by Sigma-Aldrich Chimie (St. Quentin Fallavier, France). Stock solutions of camptothecin (5 mM in dimethyl sulfoxide [DMSO]), aphidicolin (3 mM in DMSO), mevastatin (compactin; 10 mM in water), mevalonate (mevalonic acid lactone; 200 mM in water), and nocodazole {methyl-(5-[thienylcarbonyl]-1H-benz-imidazol-2-yl)carbamate; 30 mM in DMSO} were stored frozen in aliquots and diluted immediately prior use.

Cytotoxicity assay. Cells were seeded in 96-well culture microplates at 2.5×10^3 to 5×10^3/well in 100 μl for 24 h. After removal of culture medium, cells were washed with phosphate-buffered saline (PBS) and then exposed to DNA-damaging treatment. Then cells were incubated in fresh medium for 48 h. Cytotoxicity was measured by the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] metabolic dye assay. Briefly, MTT (0.3 mg/ml of culture medium) was added, and the plates were incubated for 2 h at 37°C. After centrifugation, the medium was discarded and the resulting formazan crystals were solubilized in DMSO under gentle shaking. The plates were scanned at 570- and 690-nm wavelengths with a Titertek Multiskan Plus plate reader. Cytotoxicity was expressed as the ratio of the absorbance of treated versus untreated cells. Results were expressed as curves from which the 50% inhibiting dose (ID_{50}; for UVC irradiation) or the 50% inhibiting concentration (IC_{50}; for drug treatment) was determined (50% reduction of the control absorbance value).

Whole cell extract. After two washes with PBS, cells were scrapped and collected in ice-cold PBS. The cells were counted under the microscope and then centrifuged. The cell pellet was mixed with lysis buffer (62.5 mM Tris HCl [pH 6.8], 4 M urea, 10% glycerol, 2% SDS, bromophenol blue, 5% β-mercaptoethanol) in 300 μl per 3×10^6 cells. The lysates were sonicated for 15 s (Branson Sonifier 250) and denatured for 5 min at 65°C, and proteins were directly loaded onto gels for SDS-PAGE.

Western blot analysis. The monoclonal antibody against the p32 subunit of RPA (9H8; also called 34A) was as already reported (42). Protein extracts were loaded onto SDS–12% polyacrylamide gels, transferred to a polyvinylidene difluoride membrane (Amersham), and hybridized with RPA32 monoclonal antibody (1/5,000). After incubation with a goat anti-mouse horseradish peroxidase-coupled antibody (1/20,000) (Pierce), the membrane was developed for antibody binding by the enhanced chemiluminescent Super Signal CL:horseradish peroxidase substrate system (Pierce) and XAR-5 films (Kodak) were used to visualize the protein bands. For data presentation, films were scanned and processed with Adobe Photoshop 3.0 software.

Cell cycle analysis. Before sampling, cells were incubated for 1 h with 30 μM bromo-2′-deoxyuridine (BrdU) in culture medium. After trypsinization followed by two washes in PBS, cells were fixed in 70% ice-cold methanol for 1 h. After centrifugation, cell pellet was resuspended in 2 N HCl at 25°C for 25 min, washed twice with PBS in the presence of bovine serum albumin (BSA; 1%), and centrifuged. Then the cell pellet was incubated in 0.1 ml of anti-BrdU fluorescein isothiocyanate-conjugated antibody (1/20; Caltag) for 30 min at 25°C and washed two times in PBS-BSA as described above with Tween 20 (0.5%). After 15 min of incubation in RNase A (1 mg/ml of PBS) and propidium iodine (10 μg/ml of PBS), the samples were analyzed in a FACScan (Becton Dickinson). After appropriate gating, 10^4 events were analyzed using LysisII software (Becton Dickinson). The number of events corresponding to nonspecific interactions of anti-BrdU antibodies was determined in a control experiment without BrdU incorporation and subtracted in each case.

RESULTS

UVC-induced RPA32 phosphorylation in NER mutant cells. First, the kinetics of RPA32 phosphorylation following UVC irradiation (post-UVC) was established in the control MRC5 cell line, derived from a healthy individual (Fig. 1A). Western blotting of cell lysates using a specific monoclonal antibody showed two slower-migrating bands accumulating gradually between 4 and 6 h post-UVC with no further changes from 8 to 16 h post-UVC. This reduced mobility is typical of the UVC-induced RPA32 forms observed under identical electrophoretic conditions and that have been characterized as bearing the highest number of phosphorylation sites (77). In addition, a similar time course of RPA32 phosphorylation has been observed under identical DNA-damaging conditions in extracts from HeLa cells (14).

We wanted to determine the role of NER in the pathway leading to RPA32 hyperphosphorylation following cell exposure to UVC light. To address this point, we analyzed the UVC-induced RPA32 phosphorylation in extracts from UVC-irradiated human cell lines deficient in the NER process. We used cell lines representative of the human cancer-prone inherited disease XP exhibiting a defect in the early lesion recognition (complementation groups A and C) and incision steps (group G) of NER. The MRC5 cell line and an XP-A cell line transfected with the normal *XPA* cDNA were included as a control.

The UVC sensitivity of control and XP cell lines under the

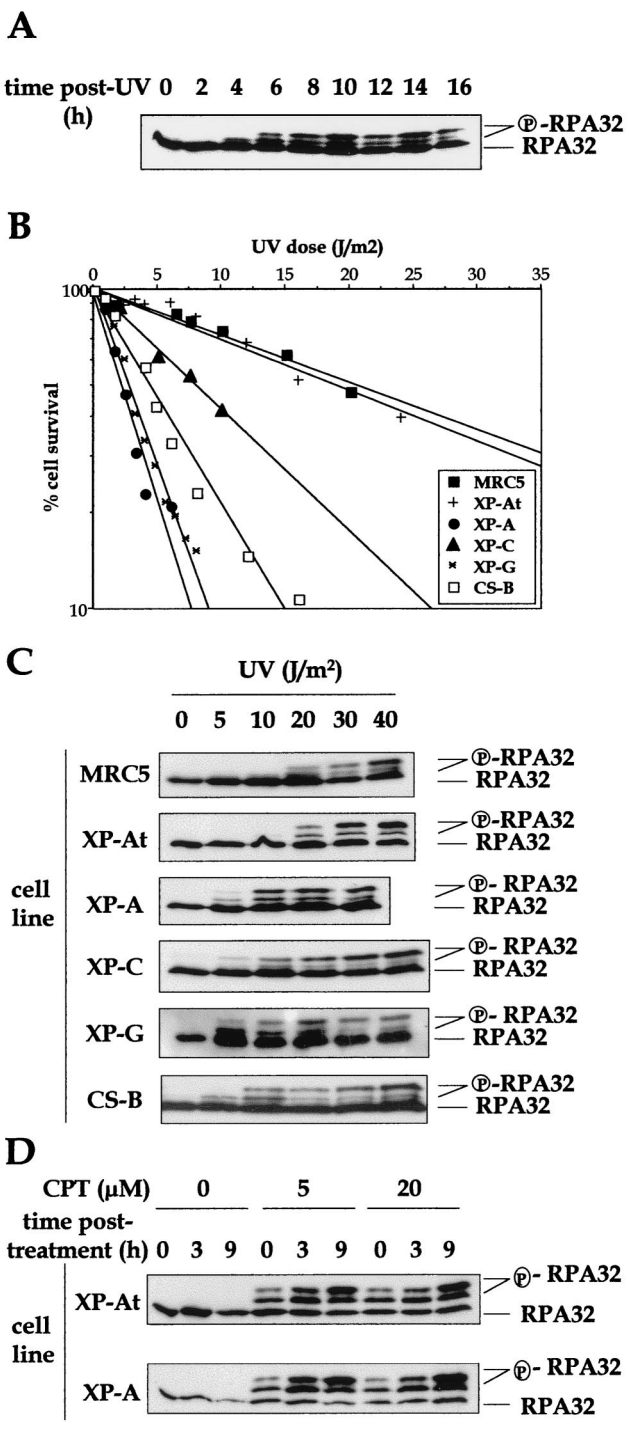

FIG. 1. UVC irradiation of nonsynchronized cells. (A) Time course of UVC-induced RPA32 hyperphosphorylation. Exponentially growing MRC5 cells were irradiated with a single dose of UVC (40 J/m^2). Briefly, after removal of culture medium, cells were washed with PBS and then exposed to UVC irradiation (254 nm) with a germicidal lamp (Bioblock, Illkirch, France) at a fluence of 0.5 J/m^2/s. Immediately after irradiation, fresh medium was added and cells were postincubated in culture medium for the indicated time period. After cell lysis, whole cell protein extracts were loaded onto an SDS–12% polyacrylamide gel, and the RPA32 phosphorylation pattern was analyzed by Western blotting with monoclonal antibody 9H8 followed by enhanced chemiluminescence detection. The two major hyperphosphorylated RPA32 forms are designed by P-RPA32. (B) Typical UVC survival of control and repair-deficient cells. Cells were UVC irradiated as described above with increasing UVC fluences. After 48 h of postincubation in culture medium, cytotoxicity was measured by the MTT assay. The absorbance value obtained for untreated control cells was standardized at 100%, and cytotoxicity was expressed as the ratio of the absorbance of treated versus untreated cells. (C) Dose response of UVC-induced RPA32 phosphorylation in control and XP cells. MRC5, XP-At, XP-A, XP-C, XP-G, and CS-B cells were treated with increasing doses of UVC as indicated, and the cells were postincubated for 14 h at 37°C. After cell lysis, RPA32 phosphorylation pattern was analyzed in whole cell protein extracts as for panel A. The two major hyperphosphorylated RPA32 forms are indicated by P-RPA32. (D) RPA32 phosphorylation induced by camptothecin in control and XP-A cells. XP-At and XP-A cells were incubated in culture medium without or with 5 or 20 μM campthotecin, as indicated, for 3 h at 37°C. After treatment, drug-containing medium was removed and replaced by fresh medium for the indicated postincubation time at 37°C. The analysis of RPA phosphorylation was performed as described for panel A.

irradiation conditions used here were first determined. Exponentially growing cells were treated with increasing UVC fluences, and viability was assessed 48 h posttreatment by the MTT metabolic dye assay. The data from a typical survival experiment are shown in Fig. 1B. For each cell line, the mean ID_{50} was calculated from the ID_{50} determined graphically from the survival curves of at least three independent experiments. Both control MRC5 and XP-At cell lines have similar ID_{50}s (19.3 ± 2.9 and 17.4 ± 1.5 J/m^2, respectively) as described previously (76). In sharp contrast, the ID_{50} of XP cell lines was much lower (2.2 ± 0.2, 2.7 ± 1.3, and 8.9 ± 1 J/m^2 for XP-A, XP-G, and XP-C cell lines, respectively). CS-B cells deficient in transcription-coupled repair (see below) were also UVC sensitive (ID_{50} = 4.6 ± 0.9 J/m^2). These values were in agreement with published data on the UVC sensitivity of identical (76) or equivalent (16) cell lines.

Because the UVC sensitivities of the various cell lines used were different, we performed a dose-response experiment on a range of UVC fluences from 5 to 40 J/m^2. As shown in Fig. 1C, RPA32 phosphorylated forms were detected in extracts from all the cell lines 14 h after UVC irradiation, with an identical pattern at the highest UVC dose. However, the dose response for RPA32 phosphorylation was different since both control cell lines exhibited a significant phosphorylation at 20 J/m^2, whereas RPA32 was already phosphorylated at 5 J/m^2 in the three XP cell lines. In a separate experiment, we established that the time course of RPA32 phosphorylation was the same in control and XP cells after UVC irradiation and that the low dose delivered to control cells did not induce a transient phosphorylation that would not have been detected at later time (data not shown).

To rule out an intrinsic property of XP cells as the basis for their difference with control cells in the UVC dose dependence of RPA32 phosphorylation, it was necessary to treat control and XP cell lines with a DNA-damaging agent producing lesions not repaired by the NER pathway. RPA32 phosphorylation following cells treatment with camptothecin has been documented (62, 69). Camptothecin produces topoisomerase I-linked DNA breaks which are likely converted into replication-mediated DNA double-strand ends repaired by recombinational pathways (for a review, see reference 56). As expected, we found the same sensitivity of XP-At and XP-A cells after a 3-h camptothecin treatment (ID_{50}s of 5.3 and 6.5 μM, respectively [data not shown]). A time course of RPA32 phosphorylation following camptothecin treatment with two concentrations of the drug was then performed in both cell lines (Fig. 1D). The results indicated that camptothecin was actually a potent RPA32 hyperphosphorylation inducer but, in contrast to UVC light, showed no difference in dose response between the control and XP cells at any time posttreatment.

UVC-induced RPA32 phosphorylation in TCR-proficient and -deficient cells. UVC photoproducts in DNA are removed

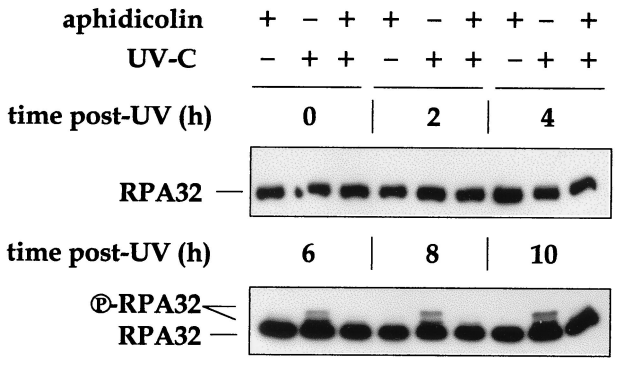

FIG. 2. Time course of UVC-induced RPA32 phosphorylation in the presence of aphidicolin. MRC5 cells were not treated or treated with 0.2 μM aphidicolin for 30 min. After the culture medium was removed, cells were irradiated with UVC (40 J/m^2) and postincubated for the indicated time in the same culture medium with or without aphidicolin. Control cells treated with aphidicolin but mocked irradiated were incubated in parallel. After cell lysis, RPA32 phosphorylation pattern was analyzed in whole cell protein extracts by Western blotting.

faster from RNA polymerase II-transcribed genes and transcribed strands (TCR) in comparison with the whole genome (global repair) (50). A role of persisting UVC lesions in transcribed genes has been found by several groups in the case of the UVC-induced nuclear accumulation of p53 protein (7, 22, 48, 75).

Since RPA interacts with several transcription factors including p53, a role of transcription arrest triggered by UVC irradiation in RPA32 phosphorylation could be questioned. If damage-dependent transcription arrest was a major signal for RPA32 phosphorylation following UV irradiation, CS cells, which are TCR deficient and unable to recover from UV-induced transcription arrest, should require lower UV dose than normal or XP-C cells, which are TCR proficient. However, our results clearly showed a different dose response between control and XP-C cells for RPA32 phosphorylation (Fig. 1C). XP-C cells that are proficient in TCR behaved like cells from the other XP groups that are totally repair deficient. In addition, we found no difference between the dose response of XP-C and CS-B (Fig. 1C) or CS-A (data not shown) cells, the two latter being selectively deficient in TCR and selectively sensitive to photoproducts in active genes. Taken together, these results allow us to exclude a prominent role of TCR and transcription arrest in RPA32 UVC-induced phosphorylation.

UVC-induced RPA32 phosphorylation in DNA replication-arrested cells. RPA is an essential component of the DNA replication apparatus (71). Since NER appeared to be unnecessary for RPA32 UVC-induced phosphorylation, we then examined the role of DNA replication in the presence of UV lesions in this process.

First, we used the DNA polymerase inhibitor aphidicolin. In a separate experiment, we determined 0.2 μM aphidicolin as the minimal drug concentration that completely inhibited DNA synthesis in MRC5 cells during a 30-min incubation, as assessed by BrdU incorporation (data not shown). Under these DNA replication-deficient conditions, the kinetics of UV-induced RPA32 phosphorylation in MRC5 cells was then followed in comparison with control conditions (Fig. 2). A minor, slower-migrating form was detected in extracts from the nonirradiated aphidicolin-treated cells, which might correspond to RPA32 phosphorylation occurring after cell exposure to inhibitors of DNA replication (47, 77). Nevertheless, Fig. 2 shows clearly that aphidicolin inhibits UVC-induced RPA32 hyperphosphorylation, which was not detected any time up to 24 h after UVC exposure (Fig. 2 and data not shown).

UVC-induced RPA32 phosphorylation in cell cycle-synchronized cells. Since aphidicolin might interfere with DNA synthesis events other than DNA replication, we decided to analyze RPA32 phosphorylation after UVC irradiation of a cell population devoid of replicating cells by treatments that do not interfere with DNA metabolism and that allow a synchronous cell cycle upon drug removal.

Cell exposure to mevastatin, an inhibitor of mevalonic acid synthesis in the cholesterol biogenesis pathway, has been reported to prevent the onset of DNA replication and to arrest cells mostly in the G_1 phase of the cell cycle; interestingly, DNA replication could be restored by the addition of mevalonate, leading to synchronous DNA replication for at least one cell cycle (43).

Thus, we performed an experiment in which both mevastatin-treated and control MRC5 cells were irradiated with a unique UVC fluence (40 J/m^2) and the time course of RPA32 phosphorylation was assessed (Fig. 3B). In parallel, the level of BrdU incorporation 1 h before sampling and cellular DNA content were simultaneously analyzed by flow cytometry (Fig. 3A). Most of the cell population accumulated in G_1 after a 48-h mevastatin treatment, and DNA replication was undetectable (Fig. 3A, time point 0 h). As expected, RPA32 hyperphosphorylated forms did not appear upon mevastatin treatment alone (Fig. 3B, time point 0 h). In the control cells, a strong decrease in BrdU incorporation beginning 2 h after UVC exposure (Fig. 3A) paralleled the accumulation of RPA32 hyperphosphorylated forms, which reached a maximum between 6 and 10 h post-UVC (Fig. 3B). In sharp contrast, only minimal phosphorylation was detectable at late times post-UVC in the nonreplicating irradiated cells (Fig. 3B).

Then, we took advantage of the reversibility of the DNA replication block induced by mevastatin and irradiated cells at various time after the addition of mevalonate; analyses of RPA32 phosphorylation 14 h post-UVC and of cell distribution in the cycle phases at the time of irradiation were conducted in parallel (Fig. 3C). Mevastatin-treated cells irradiated just upon mevalonate addition exhibited a typical inhibition of RPA phosphorylation compared with irradiated-unblocked cells (Fig. 3C, lanes 1 and 2). As expected, mevalonate allowed the cells to enter the S phase. In contrast, mevalonate-treated cells had regained the capacity to phosphorylate RPA32 following UVC irradiation 3 h after mevalonate addition. Notably, the most abundant hyperphosphorylated forms were detected for an UVC irradiation delivered 6 h after mevalonate addition, corresponding to the maximal fraction of S-phase cells after release from the G_1 arrest (44% of replicating cells) (Fig. 3C, lane 6). As control, unirradiated mevalonate-treated cells exhibited no significant RPA hyperphosphorylation at any time.

To rule out a side effect of mevastatin, we repeated the UVC irradiation experiment on MRC5 cells synchronized with another technique, M-phase arrest in the presence of nocodazole. As shown in Fig. 4A, exposure of MRC5 cells to 1.25 μM nocodazole for 16 h led to the accumulation of cells in G_2-M with almost undetectable cells in S phase. However, further incubation in the absence of drug allowed the cells to release from the G_2-M block. After about 10 h, the cells entered synchronously S phase, which reached a peak between 12 and 18 h after drug removal. Under these synchronization conditions, we UVC irradiated cells at various time points after nocodazole removal and analyzed RPA32 phosphorylation 8 h post-UVC by Western blotting (Fig. 4B). Extracts from mock-irradiated cells exhibited a slower-migrating form of RPA above position of the nonphosphorylated RPA32 (Fig. 4B, −UV, 0 to 10 h postrelease). Since the cell samples were taken

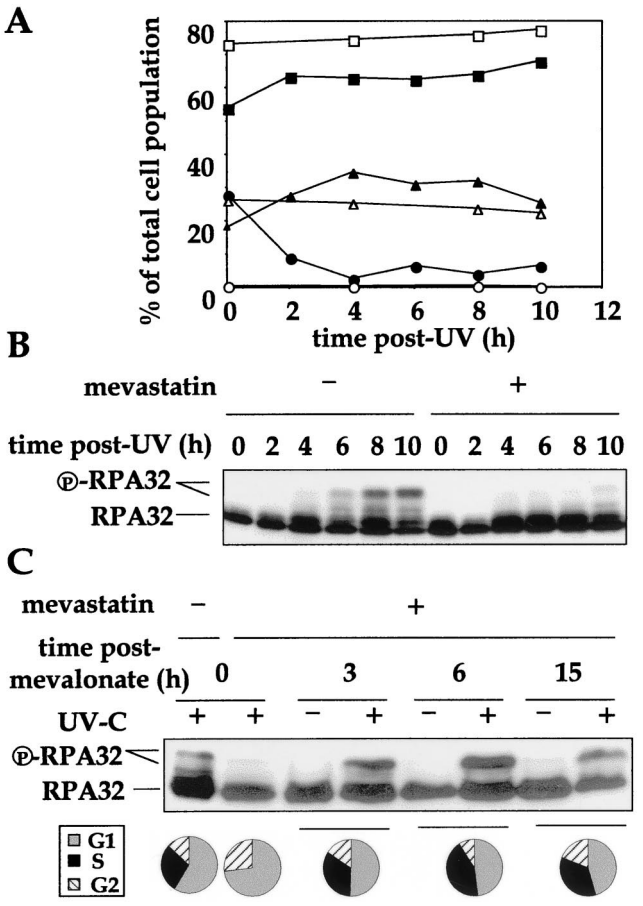

FIG. 3. Cell synchronization with mevastatin. (A) Analysis of cell cycle distribution in UVC-exposed MRC5 cells, untreated or treated with mevastatin. MRC5 cells were untreated or treated with 15 μM mevastatin for 48 h. After the culture medium was removed, cells were irradiated with UVC (40 J/m^2) and postincubated for the indicated time in culture medium with or without mevastatin. Two cell samples for each time point were run in parallel. One sample for each time point was incubated with BrdU 1 h before the end of post-UVC incubation time and analyzed for cell cycle distribution by FACScan. The curves show the percentages of cells in each phase of the cell cycle calculated from dot plots of cell cycle distribution and BrdU incorporation. Squares, circles, and triangles represent G_1, S, and G_2-M phases of the cell cycle, respectively. Open symbols represent mevastatin-treated cells, and closed symbols represent untreated cells. (B) Time course of UVC-induced RPA32 phosphorylation in MRC5 cells untreated or not treated with mevastatin. After lysis of one cell sample for each time point of the experiment described for panel A, RPA32 phosphorylation pattern was analyzed in whole cell protein extracts by Western blotting. (C) Analysis of UVC-induced RPA32 phosphorylation in MRC5 cells synchronized in S phase. MRC5 cells were untreated or treated with 15 μM mevastatin for 48 h. Then the medium was removed, and the cells were further incubated in fresh medium containing 2 mM mevalonate for 3, 6, and 15 h as indicated. Mevalonate was not added in control cells (0). Two cell samples for each time point were run in parallel. One sample for each time point was incubated with BrdU for 1 h before cell cycle analysis as described for panel A. Pie diagrams showed the percentage of cells in each phase of the cell cycle calculated from dot plots of cell cycle distribution and BrdU incorporation obtained by FACScan analysis. After removal of the medium in one cell sample for each time point, cells were irradiated with UVC (40 J/m^2; +) or mock irradiated (−) and then postincubated in the same medium for 14 h. After cell lysis, the RPA32 phosphorylation pattern was analyzed in whole cell protein extracts by Western blotting.

8 h after the time point indicated, mock-irradiated samples corresponded to cells that were between the early G_1-S transition (time point 0 + 8 h) and the end of S phase of the cell cycle (time point 10 + 8 h) at the time of sampling (Fig. 4A). Thus, the phosphorylated RPA form present in extracts from mock-irradiated cells probably corresponded to the normal cell cycle-dependent phosphorylation observed during G_1 to S transition and persisting through S phase (21, 26, 27). In addition to this form, samples from UV-irradiated cells contained hyperphosphorylated RPA32 (Fig. 4B, +UV, 0 to 18 h postrelease). This hyperphosphorylated from was faintly observed in cells irradiated between 0 and 10 h after nocodazole removal (Fig. 4B), corresponding to cell populations containing less than 10% S-phase cells at the time of UVC treatment (Fig. 4A). In contrast, substantial hyperphosphorylated RPA32 was present in extracts from synchronized cells that were irradiated between 12 and 18 h after release from the G_2-M arrest, corresponding to cell populations with a large fraction of S-phase cells (from 32% [time point 18 h] up to 78% [time point 14 h] of total cells at the time of UVC irradiation). To check whether the S-phase-dependent UV-induced phosphorylation of RPA32 is persistent beyond the first round of S phase, we performed a complementary experiment in which later time points were examined after release from nocodazole (Fig. 4C and D). Although cell synchrony was less pronounced during the second cell cycle following nocodazole removal, the same correlation between the onset of S phase and significant levels of UVC-induced RPA32 phosphorylation was observed during the second cell cycle (time points 20 to 40 h in samples from UV-irradiated cells).

Finally, the use of nocodazole allowed us to compare the kinetics of RPA32 phosphorylation following UVC irradiation of nonsynchronized cells with cells mainly in S phase. Figure 5 shows that the onset of RPA32 phosphorylation occurs between 0.5 and 1 h following UV-exposure of S-phase synchronized cells (61% of total cell in this experiment), while the onset occurs 6 to 8 h after UV irradiation in asynchronous cells.

DISCUSSION

Hyperphosphorylation of RPA32, the 32-kDa subunit of the single-stranded DNA-binding protein RPA, has been observed as one of many UVC-induced phosphorylation events (14, 47). In addition, RPA32 has been shown to be phosphorylated both in a cell cycle-dependent manner (21, 26) and in response to various other DNA-damaging treatments (12, 14, 47, 62, 69). The heterotrimeric RPA complex is required for major cellular processes like recombination, DNA replication, and removal of DNA damage by NER (for a review, see reference 71). Here, we have addressed the question of which signal triggers RPA32 hyperphosphorylation following UVC irradiation in human cells.

From the present study, we can exclude a direct role of NER DNA intermediates in the process leading to RPA32 UVC-induced phosphorylation since hyperphosphorylation was observed in cells completely deficient for the NER pathway. In addition, we establish that UVC-sensitive XP and CS cell lines required lower UVC doses to induce RPA32 phosphorylation in comparison with control repair proficient cell lines. In contrast, both control and XP cell lines exhibit the same dose response to the non-NER substrate lesion-inducing agent camptothecin. Thus, it can be concluded that UVC-induced RPA32 phosphorylation is a DNA lesion-dependent process. This is in contrast with early UVC-induced events like c-*fos* transcription which have been shown to be mediated via cytoplasmic signal transduction and for which no dose difference between control and XP cells was found (57, 58). The delayed kinetics of RPA32 UVC-dependent phosphorylation is also in agreement with a DNA damage-dependent process since, even in repair-proficient cells, half of the most abundant class of UVC lesions persists in DNA beyond 4 h after a mild-dose irradiation (reference 29 and references therein).

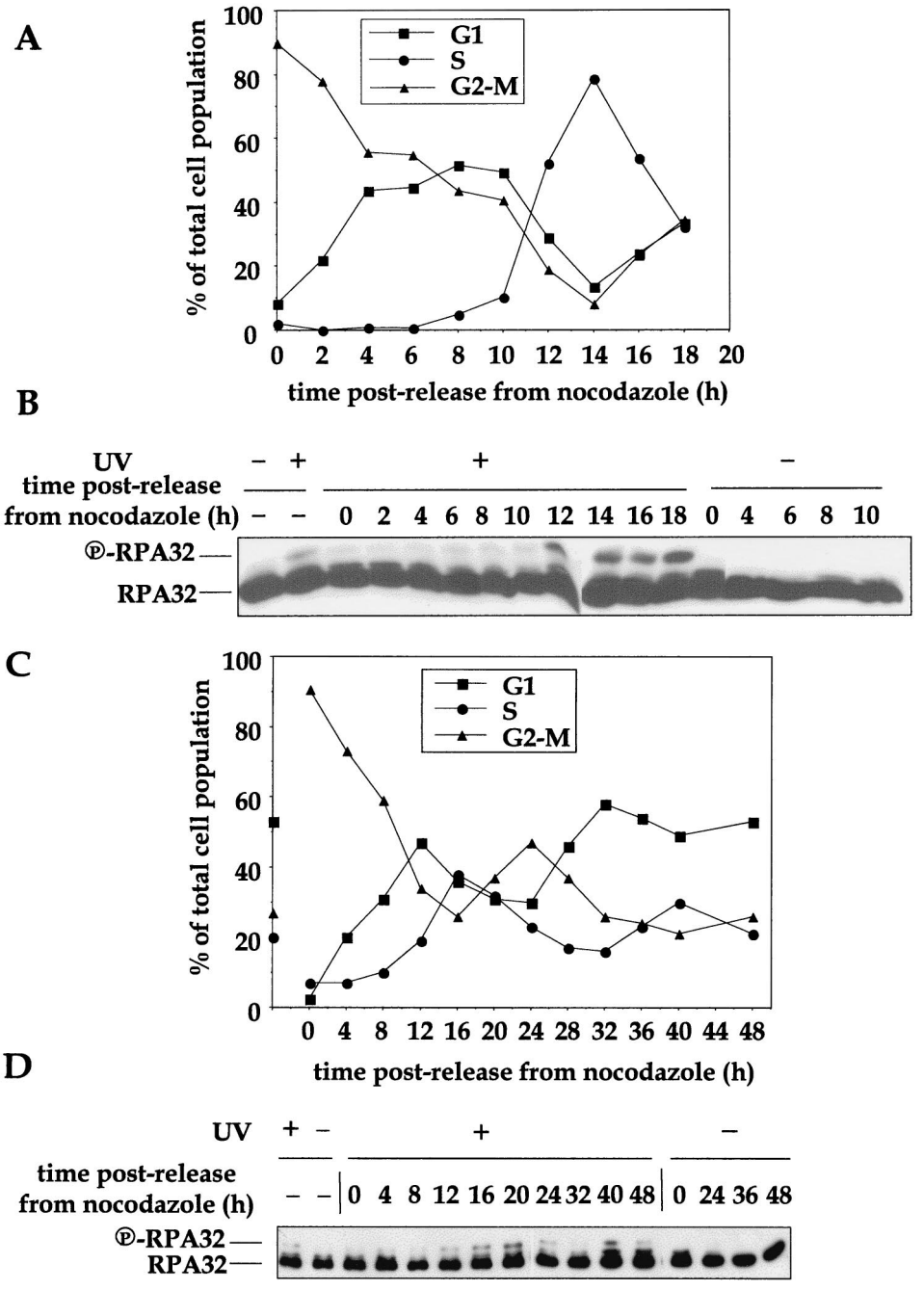

FIG. 4. Cell synchronization with nocodazole. MRC5 cells were treated with 1.25 μM nocodazole in culture medium for 16 h. After the medium was removed, the cells were rinsed twice with PBS and further incubated in fresh medium without drug for a time interval as indicated. Two cell samples for each time point were run in parallel. (A and C) Cell cycle distribution. One sample for each time point was incubated with BrdU for 1 h before cell cycle analysis as described for Fig. 3A. The curves show the percentages of cells in each phase of the cell cycle calculated from dot plots of cell cycle distribution and BrdU incorporation obtained by FACScan analysis. (B and D) Analysis of UVC-induced RPA32 phosphorylation. After removal of the medium in one cell sample for each time point, cells were irradiated with UVC (40 J/m^2; +) or mock irradiated (−) and then postincubated in the same medium for 8 h. After cell lysis, RPA32 phosphorylation pattern was analyzed in whole cell protein extracts by Western blotting. The two first lanes from the left correspond to control cells not treated with nocodazole.

The same correlation with the cell UVC sensitivity has been demonstrated for delayed UVC-responsive genes which were induced at much lower UVC fluences in XP-A cells (2 to 5 J/m^2) than in control repair-proficient cells (20 to 30 J/m^2) (7, 61). This observation can be attributed to a divergence in lesion density in DNA after UVC irradiation between normal and repair-deficient cells, due to the difference in repair capacity. Thus, although NER is not required for RPA32 UVC-induced phosphorylation, the cellular repair capacity has an indirect influence by controlling the density of persistent DNA damage.

Several studies have established a role of persisting UVC lesions in actively transcribed strands of cellular genes for UVC-induced events like nuclear accumulation of p53 protein

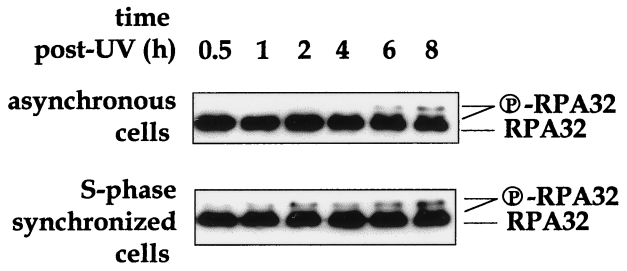

FIG. 5. Kinetics of UVC-induced RPA32 phosphorylation in S-phase synchronized cells. MRC5 cells were treated with 1.25 μM nocodazole in culture medium for 16 h. After the medium was removed, the cells were rinsed twice with PBS and further incubated in fresh medium without drug. After 12 h of incubation in fresh medium, cells were irradiated with UVC (40 J/m^2) and then postincubated in the same medium for the indicated time. In parallel, control asynchronous cells were mock treated and UV irradiated. After cell lysis, the RPA32 phosphorylation pattern was analyzed in whole cell protein extracts by Western blotting.

(7, 22, 48, 75) or induction of collagenase and metallothionein genes (7). It was found that similar UVC fluences were required in control and XP-C cells (TCR proficient) for these events to occur, whereas CS cells required much lower doses. The latter were unable to repair photoproducts in transcribed DNA, leading to longer post-UV inhibition of transcription (75). Here, we establish that transcription arrest by unrepaired photoproducts has no major role in UVC-induced phosphorylation of RPA32. No difference was found in the UV doses required for RPA phosphorylation in XP-C and CS.

In contrast to NER and transcription arrest, the present study emphasizes a major role for UVC lesion-induced DNA replication arrest for signaling RPA32 phosphorylation: first, the DNA polymerase inhibitor aphidicolin abolishes UVC-induced RPA32 phosphorylation; second, we have shown by two different synchronization methods that the extent of RPA32 phosphorylation in the presence of UVC photoproducts correlates with the number of cells in S phase at the time of UV exposure; finally, the time course of RPA32 phosphorylation is greatly accelerated in cells UV irradiated in S phase.

The data presented here allow us to propose a model for UVC-induced RPA phosphorylation (Fig. 6). The damage-dependent generation of single-stranded DNA is likely to be a critical signal. Following UVC irradiation, various processes can produce single-stranded DNA regions. However, we have established that NER and transcription arrest do not lead to RPA hyperphosphorylation whereas DNA replication does. UVC photoproducts are known to interfere with DNA replication both in vitro and in vivo (for a review, see reference 29). Although some translesion synthesis might occur with eukaryotic DNA polymerases (65, 67), a majority of photoproducts are likely to block ongoing replication forks and lead to the occurrence of unreplicated regions in the vicinity of the damage, conceivably including gaps and strand breaks. Accordingly, an S-phase-dependent clastogenic effect of UVC irradiation has been reported (40). In addition, an indirect role of NER intermediates in replication arrest is unlikely since UVC-irradiated NER-deficient cells have been shown to exhibit a longer delay in progression through S phase compared to UV-irradiated repair-proficient cells (54), which would not be the case if NER was required for replication arrest in the presence of UV photoproducts. Hence, it can reasonably be hypothesized that damage-induced aberrant replication intermediates trigger the activation of specialized kinases which in turn phosphorylate RPA bound to these structures. Alternatively, having RPA persistently bound to lesions or single-stranded DNA may make RPA a better substrate for phosphorylation. Indeed, it has been shown in the SV40 replication system that the association of RPA with single-stranded DNA was necessary for its replication-mediated phosphorylation (27). In addition, other observations indicate that hyperphosphorylation of RPA32 requires RPA binding to DNA (6, 26, 31, 35, 36). Apart from binding to the single-stranded DNA intermediates generated

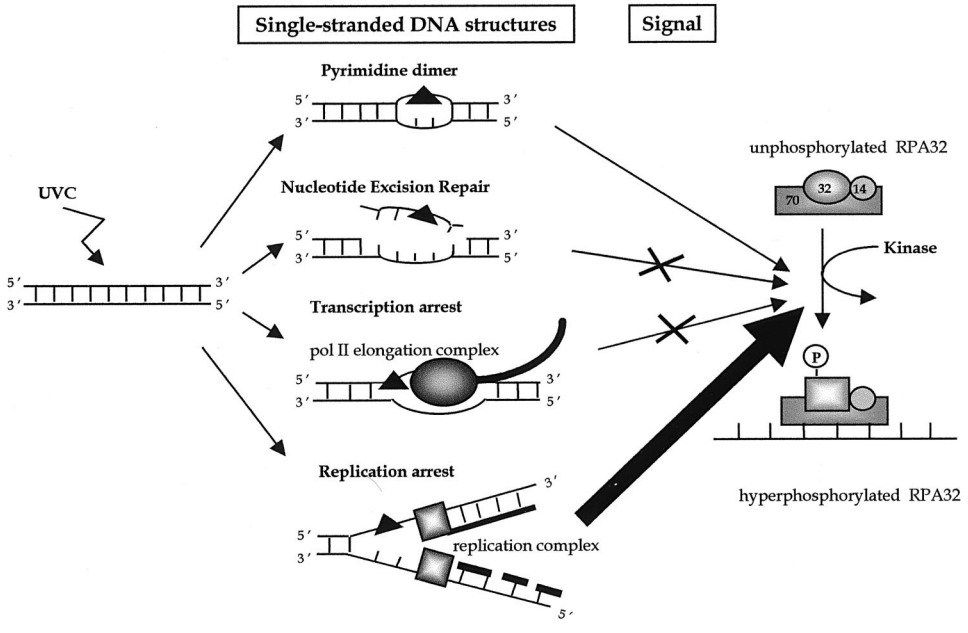

FIG. 6. Model of UVC-induced RPA32 phosphorylation. The filled triangle represents a UVC photoproduct in DNA. Single-stranded DNA can be generated either directly by local unwinding around the damage or as an intermediate in NER, transcription arrest, or replication arrest. Whereas RPA can bind to either single-stranded DNA region, replication arrest is the main signal that activates the kinase(s) responsible for RPA32 hyperphosphorylation. DNA damage by itself may represent a minor signal in this pathway.

by stalled replication forks, a minor route might also exist for RPA phosphorylation since we observed persisting though very faint hyperphosphorylation in nonreplicating UVC-treated cells (Fig. 3B and 4B). Indeed, an intrinsic affinity of RPA has been described for both cisplatin-damaged DNA (17) and UVC-damaged DNA (13). It might contribute to a marginal signal leading to RPA phosphorylation, alternative to replication block (Fig. 6).

According to our model, the rate and extent of RPA phosphorylation would then be directly dependent on the occurrence frequency of replication block in front of unrepaired photoproducts. This model fully explains the dependency of UVC-induced RPA phosphorylation that we report here on both the number of replicating cells and the extent of persistent UVC lesions. Similarly, extended G_2 arrest have been found only associated with UVC-irradiation during S phase (53). In addition, as with the RPA UVC-induced phosphorylation analyzed here, the magnitude of this effect was correlated with the cell ability to repair DNA (54), indicating a direct effect of persistent DNA damage.

Other reports are in agreement with our replication-based model for RPA damage-induced phosphorylation. Ionizing radiation (IR) does not induce RPA phosphorylation in noncycling fibroblasts (15). In addition, Shao et al. have reported recently that DNA replication is necessary for RPA phosphorylation following cell exposure to either camptothecin or IR (62), which might induce aberrant structures leading to damage-induced replication block.

Until now, the question of the kinase responsible for the UVC-induced RPA hyperphosphorylation is not resolved. The kinase responsible for the replication-mediated RPA phosphorylation in the SV40 replication system with cell extracts has been identified as the DNA-dependent protein kinase (DNA-PK) (11). DNA-PK is necessary for the repair of double-strand breaks by the nonhomologous end-joining pathway (for reviews see references 3, 38, and 39). In addition, purified DNA-PK phosphorylates the same sites in RPA32 that are phosphorylated in HeLa cells after UVC irradiation (52, 77). In yeast, the MEC1 homologue to the kinase mutated in the human disorder ataxia-telangiectasia (AT) is required for UVC- and IR-induced RPA hyperphosphorylation (12). In addition, the protein kinase activity associated with the gene product mutated in AT (ATM) phosphorylates RPA32 in vitro (30). However, while human AT cells exhibited a delayed IR-induced RPA32 phosphorylation, the UVC-induced reaction appeared normal (47). Although conflicting results were reported on the role of DNA-PK in IR-induced RPA phosphorylation (8, 28), a recent report demonstrated a role of DNA-PK following campthotecin treatment (62). Further experiments are required in order to challenge a role for kinases activated upon DNA block following UVC irradiation and with phosphorylation sites specificity similar to DNA-PK.

What could be the cellular function of RPA32 phosphorylation? We report here a temporal parallel between RPA32 phosphorylation and DNA synthesis inhibition (Fig. 3A and B). Similarly, DNA synthesis arrest in UVC-induced HeLa cells coincided with RPA32 phosphorylation (14), and the same relationship was found following camptothecin treatment (62). This suggests that DNA synthesis arrest may be related to RPA-induced phosphorylation. Indeed, the low replication competence of extracts from UVC-irradiated cells containing hyperphosphorylated RPA can be restored to normal levels by addition of unphosphorylated RPA to these extracts (14). However, another study has shown that both RPA forms had equal activity for replication in vitro (55). Thus, the question of a direct role of RPA phosphorylation in the modulation of DNA synthesis is unresolved. Similarly, although protein phosphorylation may be implicated in the regulation of NER (4), in vitro experiments with selective addition of various phosphorylated RPA32 forms to reconstituted reactions did not emphasize a role for RPA phosphorylation in this regulation (55).

Alternatively, damage-induced RPA phosphorylation might be part of a signaling pathway regulating cell cycle progression in the presence of DNA lesions. RPA has been demonstrated to associate with p53, the regulator of the major checkpoint control pathway after DNA damage (23, 33, 46). However, the interaction was disrupted after UVC damage (2). Since the hyperphosphorylated forms of RPA did not associate with p53 (2), the authors postulated that RPA phosphorylation might participate with other events to damage-induced RPA-p53 disruption, contributing to p53 accumulation and activation. Interestingly, AT cells which exhibit an abnormal IR-induced p53 activation and a DNA damage-resistant DNA synthesis are also characterized by a defect in RPA IR-dependent phosphorylation (15, 47). In addition, DNA-PK-deficient cells exhibit a reduction both in camptothecin-induced RPA phosphorylation and in DNA synthesis arrest (62). The finding of the kinase responsible for the UVC-induced RPA hyperphosphorylation and the characterization of potential defects in the DNA damage-induced checkpoints associated with its inactivation might help to elucidate the role of RPA phosphorylation in the cellular response to UVC light.

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