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H2AX phosphorylation after UV irradiation is triggered by DNA repair intermediates and is mediated by the ATR kinase

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It has been suggested that phosphorylation of the histone variant H2AX after ultraviolet light (UV) irradiation is triggered by DNA double-strand breaks induced as replication forks collide with UV-induced bulky lesions. More recently, it has been shown that UV-induced H2AX phosphorylation can also occur outside of S-phase, but the mechanism for this replication-independent induction is not well understood. In this study, we show that H2AX phosphorylation after UV irradiation is triggered by DNA repair intermediates and is induced in all phases of the cell cycle. Accumulation of DNA repair intermediates by inhibition of DNA repair synthesis resulted in a marked increase of H2AX phosphorylation in repair proficient but not repair-deficient xeroderma pigmentosum-A cells. Using chemical inhibitors of the PI(3)-like kinase family of protein kinases as well as ataxia telangiectasia mutated and Rad-3 related (ATR)-deficient Seckel syndrome cells and ataxia telangiectasia mutated-deficient ataxia telangiectasia cells, we show that the H2AX phosphorylation induced by accumulation of repair intermediates is mediated primarily by the ATR kinase. We suggest a model for UV light-induced phosphorylation of H2AX where in addition to replication blockage, DNA repair intermediates trigger H2AX phosphorylation via the ATR kinase.

Introduction

DNA damage induced by various exogenous and endogenous sources results in the activation of DNA repair pathways and the induction of a complex DNA damage-signaling cascade. The DNA damage response brings about repair, arrest of cell cycle progression or triggers certain cells to undergo apoptosis. All of these responses are thought to help counteract mutagenesis. Members of the PI(3)-like kinase family, such as ataxia telangiectasia mutated (ATM) and ataxia telangiectasia mutated and Rad-3 related (ATR), are believed to be involved in the activation of DNA damage-signaling pathways (1–3). The mechanisms of the initial activation of these kinases are not understood in detail but it is thought that they may monitor secondary effects of DNA damage such as perturbation of chromatin structure or the interference of DNA metabolic processes such as replication or transcription rather than sensing the DNA lesions directly (4–7).

The phosphorylation of the histone variant H2AX on serine 139, a form called γH2AX, has been characterized as a very early event following induction of certain types of DNA damage such as double-strand breaks (DSB) (8–11). After exposure to ionizing radiation, microscopic γH2AX 'foci' are formed presumably at sites of DSBs, which can be visualized by immunofluorescence microscopy after staining fixed cells with specific anti-γH2AX antibodies. Other DNA damage-signaling and repair proteins are also recruited to these foci and assembled into what are postulated to be multifactor repair complexes. Although the exact role of γH2AX and what γH2AX nuclear foci represent is not presently understood, it has been suggested that γH2AX plays some role in DNA repair (12–17).

In contrast to ionizing radiation, ultraviolet light (UV) does not directly induce DSBs. The primary lesions induced in DNA by UV light are cyclobutane pyrimidine dimers and 6-4 photoproducts (18). These lesions are mainly repaired by the nucleotide excision repair (NER) pathway, which also repairs chemical-induced bulky lesions. NER operates by global genomic repair (GGR), which removes lesions genome wide, and transcription-coupled repair (TCR), which removes lesions specifically from the transcribed strands of actively transcribing genes. While the initial damage recognition is different in the two pathways, both pathways involve sequential steps of excision of short damage-containing DNA segments, repair synthesis of a new DNA strand and finally DNA ligation of the new strand to the parental strand (18). Genetically inheritable NER-deficiency syndromes, such as xeroderma pigmentosum (XP) and Cockayne's syndrome (CS) have defects in one or both pathways of NER. CS cells lack TCR but have proficient GGR, XP-C cells are deficient in GGR but proficient in TCR, while XP-A cells lack both subtypes of NER and are unable to incise the DNA at sites of lesions. These cell lines provide a valuable means of studying the contribution of the individual DNA repair pathways in the activation of DNA damage response signaling.

Although some early reports failed to find H2AX phosphorylation in cells after UV irradiation (9,11,19), subsequent studies reported that γH2AX foci form in UV-irradiated cells during S-phase, suggesting that blocked replication forks trigger H2AX phosphorylation (20,21). Interestingly, γH2AX is not induced at early times after UV irradiation in XP-A cells, which are deficient in NER, suggesting a role for DNA repair factors in the triggering of H2AX phosphorylation (22) and in the activation of the ATR-signaling pathway in the S-phase of the cell cycle (23). More recent studies have shown induction of H2AX phosphorylation in non-S-phase cells after UV, but the mechanism responsible for this replication-independent induction is unknown but may involve NER processing (24–27).

In the present study, we further investigated the kinetics and mechanisms of UV-induced H2AX phosphorylation in human primary fibroblasts. We show that UV-induced γH2AX formation is not limited to replicating cells but also occurs in the G_1 and G_2/M phases of the cell cycle. The contribution of GGR in inducing γH2AX may be greater than that of TCR, since GGR-deficient XP-C cells show much lower levels of γH2AX than GGR-proficient, TCR-deficient CS-B cells. Finally, we show evidence that the ATR kinase plays a major role in H2AX phosphorylation, resulting from accumulation of repair intermediates after UV irradiation.

Abbreviations: araC, cytosine arabinoside; ATR, ataxia telangiectasia mutated and Rad-3 related; ATM, ataxia telangiectasia mutated; CS, Cockayne's syndrome; DSB, double-strand breaks; GGR, global genomic repair; HU, hydroxyurea; MEM, modified Eagle medium; NER, nucleotide excision repair; PBS, phosphate-buffered saline; TCR, transcription-coupled repair; UV, ultraviolet light; UVC, ultraviolet light C; XP, xeroderma pigmentosum.

Materials and methods

Cell culture

Immortalized human diploid fibroblasts derived from normal (NF), XP-A (GM 1630), XP-C (GM 00671) and CS-B (1098B) patients (purchased from Coriell Cell Repositories) were grown as monolayers on culture dishes or cover slips in modified Eagle medium (MEM) supplemented with 10% fetal bovine serum (Hyclone) and antibiotic/antimycotic (Gibco BRL). All cell lines were maintained at 37°C and 5% CO_2. For serum starvation experiments, cells were plated with MEM medium and allowed to grow to 60–70% confluence. The media was then removed and replaced with serum-free MEM and cells were allowed to incubate for an additional 72 h. The cells were then treated and allowed to recover in serum-free MEM. Exponentially growing cells were plated 1 day prior to treatment. ATR-Seckel lymphoblasts (DK0066) were a kind gift from Dr Judith Goodship, Newcastle University, and the AT

lymphoblasts (1526) and wild-type lymphoblasts (536) were a kind gift from Chris Canman, University of Michigan. All lymphoblast cell lines were grown in RPMI with 15% fetal bovine serum and streptomycin/penicillin.

Reagents and treatments

Ultraviolet light C (UVC) irradiation (254 nm) of cells was performed at room temperature at a fluence of 0.6 J/m^2/s as measured by a UV radiometer (UVX radiometer, UVP) and cells were then allowed to recover at 37°C. Aphidicolin, hydroxyurea (HU), cytosine arabinoside (araC), caffeine and wortmannin (Sigma) were used at indicated concentrations. Aphidicolin or hydroxyurea/araC was added to the media 30 min before UVC irradiation and these drugs were kept in the media throughout the recovery period. Where indicated, wortmannin or caffeine was added to media along with aphidicolin.

Immunocytochemistry

Cells grown on cover slips were exposed to UVC light and allowed to recover for 6 h at 37°C. The cells were then washed with phosphate-buffered saline (PBS) and fixed in ice-cold methanol/acetone (1:1) and stored at −20°C for at least 30 min. The fixed cells were washed twice in PBS and incubated for 2 h with anti-phospho-H2AX (ser 139) mouse monoclonal antibodies (Upstate Biotechnology, 05-636). The antibodies were washed off and cells were rinsed thrice in 5 g bovine albumin and 500 µl Tween 20/l of PBS and incubated for 1 h in the dark with secondary FITC-conjugated anti-mouse IgG antibody (Sigma). Cells were then rinsed twice in 5 g bovine albumin and 500 µl Tween 20/l of PBS and the cover slips were mounted on glass slides using a drop of Vectashield containing DAPI (Vector Laboratories). Images were captured using a Zeiss Akioskop fluorescent microscope with Plan-NEUFLUAR 63X/1.25 oil lens and a CoolSnapPro (Media Cybergenics) digital camera with associated software.

Western blotting

Cells were rinsed with PBS, detached by scraping and collected by centrifugation. Cells were lysed by boiling for 5 min in loading buffer [2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, 0.05% bromophenol blue and 62.5 mM Tris (pH 6.8)]. Samples were subsequently sonicated for 12 s using a microtip (Heat Systems-Ultrasonics) and protein concentrations were quantified using the Bio-Rad protein assay. Approximately 30 µg of protein was loaded per lane and the samples were run on a 15% polyacrylamide gel. Following sodium dodecyl sulfate–polyacrylamide gel electrophoresis, proteins were electrophoretically transferred to Immobilin-P transfer membranes (Millipore) using Trans-blot semi-dry electrophoretic transfer cell (Bio-Rad). Blots were blocked in TBST containing 5% milk for 1 h at room temperature, rinsed twice in TBST before incubation with anti-phospho-H2AX (ser 139) antibody (Upstate, 05-636) overnight at 4°C on a slow rocking platform. The blots were then rinsed three times in TBST and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody for 1 h at 20°C. Following three rinses in TBST, the protein bands were detected by Enhanced chemiluminescence (SuperSignal CL-HRP Substrate System, Pierce) and X-ray film (Biomax AR, Amersham). The western blots in Figure 6 were imaged with Odyssey infrared imaging system (Licor Biosciences) using Immobilon-FL membranes (Millipore) and goat anti-mouse fluorescent secondary antibody IRDye 680 (Licor Biosciences). Images were captured and analyzed using the Odyssey 2.1 software. The efficiency of total protein transfer was assessed by staining the blots with anti-β-actin antibodies as well as staining blots with Coomassie Brilliant Blue after the completion of the protein determinations.

Flow cytometry

Cells were collected by trypsinization and centrifugation, rinsed with PBS and fixed by adding drop-wise ice-cold 70% ethanol while vortexing. Fixed cells were stored at −20°C for up to 1 week. On the day of analysis, the ethanol was aspirated, cells were rinsed in PBS and incubated in anti-phospho-H2AX antibody diluted 1:100 in HBT (PBS, 5% fetal bovine serum, 0.5% Tween 20) for 2 h at room temperature. Cells were rinsed and suspended in FITC-conjugated anti-mouse IgG antibody diluted 1:20 in HBT for 45 min in the dark. Cells were then rinsed and suspended in 1× propidium iodide containing RNase at 4°C and quantified for FITC (γH2AX) and propidium iodide (DNA content) using a Coulter Elite ESP cell sorter.

Results

Ultraviolet light C irradiation induces γH2AX nuclear foci in human fibroblasts

It has been previously shown that UV light triggers the phosphorylation of H2AX in human fibroblasts (19,22,25,27) and in a number of human cancer cell lines (20,24). Since UV light does not directly induce DSB in DNA, it has been suggested that H2AX phosphorylation may be triggered by DSBs formed as a result of replication blockage at sites of UV-induced lesions (19–21).

To further investigate the mechanism of H2AX phosphorylation following UV irradiation, we subjected exponentially growing normal human fibroblasts cells to 5 or 20 J/m^2 of UVC irradiation (254 nm) and the presence of nuclear γH2AX foci was determined 6 h later by immonocytochemistry. By using specific anti-γH2AX antibodies and immunocytochemistry, the presence of discrete subnuclear spots or 'nuclear foci' which presumably represent hundreds or thousands of γH2AX molecules can be revealed. Using this approach, we found that while unirradiated control samples had a few cells staining positive for γH2AX, UV irradiation caused a dose-dependent increase in the number of cells with γH2AX foci. Following the lower dose of 5 J/m^2 we saw a partial response where some cells stained positive while others did not, although all cells were exposed to the same dose of UV irradiation. Following exposure of the cells to 20 J/m^2, almost all cells stained positive for γH2AX. Interestingly, the intensity of γH2AX staining differed between the exposed cells, which is in agreement with findings from a recent study (25). However, in contrast to that study, we did not notice any pan-nuclear staining in any of the cells analyzed but rather the staining was concentrated to nuclear foci throughout the nuclei (Figure 1B). We do not believe that the phosphorylation of H2AX that we observed 6 h after 5 or 20 J/m^2 was due to the induction of apoptosis since we previously have shown that only ∼5 or 10% apoptosis is induced in human fibroblasts following 5 or 20 J/m^2 of UV irradiation, respectively (28).

Time and dose-dependent induction and disappearance of H2AX phosphorylation following UV irradiation

We next analyzed the kinetics of γH2AX formation after exposure to three different doses of UV light using phospho-specific anti-γH2AX antibodies and western blot. As can be seen in Figure 2, γH2AX is clearly detected within the first 2 h of irradiation. The level of γH2AX peaked at 2 h following exposure to 5 J/m^2, while it peaked at 6 h following exposure to 10 J/m^2. Following exposure to 20 J/m^2 of UVC, γH2AX was induced to levels 100-fold higher than control cells and this high level was sustained throughout the 12 h time period. These results show that both the induction and disappearance of H2AX phosphorylation following UV irradiation is dose dependent.

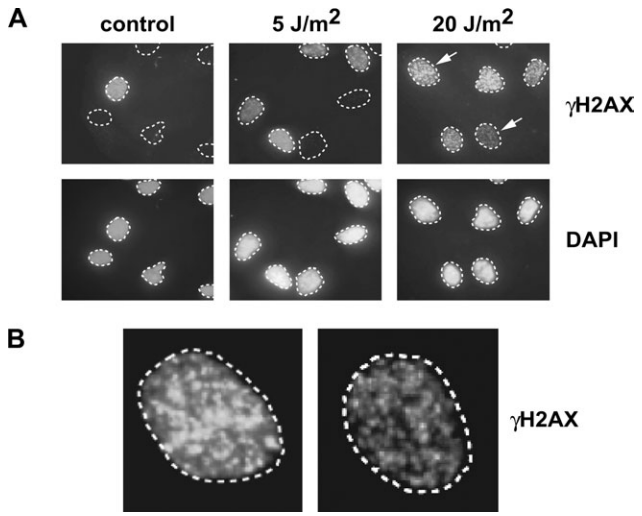

Fig. 1. UVC induces γH2AX nuclear foci in human fibroblasts. (**A**) Immunocytochemistry of normal human fibroblasts mock treated or exposed to 5 or 20 J/m^{2}UV light and allowed to recover for 6 h. Cells were then fixed and stained for γH2AX (top) or DNA (bottom). The nucleus is outlined by dashed line. (**B**) Enlarged image of the cells indicated by arrows shown in (**A**).

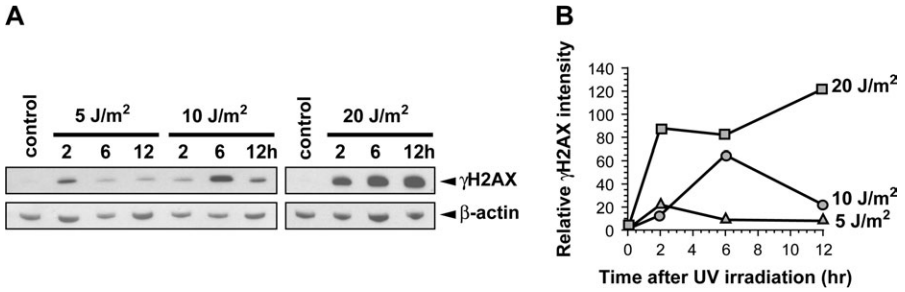

Fig. 2. Time- and dose-dependent increase of γH2AX after UV irradiation. (**A**) Western blot analysis showing γH2AX induction in normal fibroblasts after indicated doses and times after UV exposure. β-actin is shown as a loading control. (**B**) Quantitative analysis of band intensity from the blots shown in (**A**). Bands were quantified using NIH Image software and normalized to the β-actin loading control band.

It is expected that ∼25% of the cyclobutane pyrimidine dimers are removed in human fibroblasts during the first 2 h after UV irradiation with 10 J/m^2, while 60 and 70% are removed by 6 and 12 h, respectively (29). Since many cells are destined to undergo apoptosis following exposure to 20 J/m^2, but not following exposure to 10 or 5 J/m^2 (28), the ability of cells to reverse H2AX phosphorylation within 12 h may be predictive of survival. The high level of sustained γH2AX at 12 h following exposure to 20 J/m^2 is not probably due to an activated apoptotic pathway inducing DNA fragmentation since the first signs of apoptosis do not appear until ∼36 h after exposure in these cells (data not shown).

UV-induced H2AX phosphorylation is cell cycle independent

The results showing that almost all cells stained positive for γH2AX following 20 J/m^2 (Figure 1A) suggested to us that H2AX phosphorylation can occur independently of replication since only a small fraction of the cells are expected to be in S-phase at the time of irradiation or during the post-incubation period. To investigate the influence of cell cycle position on the induction of γH2AX following UV irradiation, we employed flow cytometry of fixed cells stained with anti-γH2AX antibodies and the DNA stain propidium iodide. Inspection of flow diagrams revealed that UV irradiation induced γH2AX in all phases of the cell cycle (Figure 3A). Plotting the dose and time-dependent induction of H2AX phosphorylation as a function of cell cycle phase, it became obvious that there were no marked difference between the phases. These findings are consistent with previous reports (24–26). The S-phase cells had a slightly higher basal level of H2AX phosphorylation than the other phases so when the data are normalized to the background levels of H2AX phosphorylation, the UV-induced H2AX phosphorylation in S-phase cells do not appear to be higher than in other cell cycle phases. To make sure that the induction of H2AX phosphorylation observed in G_1 cells is not due to the redistribution of cells that traversed S-phase during irradiation and made it through mitosis back into G_1 before cells were collected, we used nocodozole to trap cells in mitosis. Thus, the cells positioned in the G_1 phase at collection must have been there throughout the post-incubation time. When we specifically examined the G_1 population in the nocodazole-treated cells, we found that they still induced a robust H2AX phosphorylation following UV irradiation. That asynchronously growing cells induce H2AX phosphorylation in the G_1 phase of the cell cycle in response to UV irradiation is contrast to a recent publication showing that H2AX phosphorylation in growing cells occur specifically in S-phase (27). Finally, we analyzed UV-induced γH2AX formation in serum-starved normal fibroblasts as well as BrdU-labeled cells. Serum starvation caused cells to accumulate in G_1 (90–95% by flow cytometry) and these cells also showed dose- and time-dependent increase of γH2AX in the G_1 cell population after UV irradiation as determined by western blotting and flow cytometry (data not shown). Furthermore, BrdU labeling of replicating cells followed by UV irradiation and recovery for 6 h showed γH2AX induction in both BrdU-positive as well as BrdU-negative cells (data not shown). Taken together, our results show a time- and dose-dependent phosphorylation of H2AX and that following lower exposures to UV light, cells can reverse this phosphorylation in a time-dependent manner. Furthermore, our results and those of others showing cell cycle-independent induction of γH2AX (24–27) suggest that in addition to responding to replication-induced DSBs at sites of UV lesions, other cellular mechanisms to induce H2AX phosphorylation must be at work following UV irradiation.

Role of DNA repair processing in UV-induced γH2AX formation

Repair of UV-induced lesions occurs by NER. Single-stranded DNA intermediates are formed during NER by repair-excision of a short stretch of DNA containing the UV lesions (18). To test whether DNA repair intermediates may be a trigger for the phosphorylation of H2AX, we accumulated single-stranded DNA repair intermediates by blocking DNA repair synthesis following UV irradiation using the DNA polymerase inhibitors aphidicolin or HU/araC (30–33). The rationale for these experiments was that if DNA repair intermediates trigger γH2AX formation, the use of the DNA repair synthesis inhibitors should accumulate these intermediates and result in a longer half-life of γH2AX in cells. Normal human fibroblast (NF) was exposed to 5 J/m^2 UV irradiation with or without pretreatment with aphidicolin, araC, HU or the combination of HU and araC for 30 min and induction of γH2AX was evaluated at 6 h after irradiation. It can be seen in Figure 4A that the inhibitors or 5 J/m^2 UV light alone only marginally increased H2AX phosphorylation. However, when UV light was combined with either aphidicholin or HU/araC, induction of γH2AX was greatly increased.

To rule out that this dramatic increase in UV-induced γH2AX formation by inhibition of DNA polymerases was not due to effects these agents may have on cells undergoing replication, the experiments were repeated using confluent, serum-starved cells. We found that the pretreatment with either HU/araC or aphidicolin enhanced UV-induced γH2AX formation in growth-arrested cells similarly to exponentially growing cells (Figure 4B). Taken together, our results suggest that DNA repair-induced intermediates may play an important role in the induction of H2AX phosphorylation after UV irradiation.

Relative roles of TCR and GGR in UV-induced H2AX phosphorylation

UV-induced DNA lesions can be repaired by either TCR or GGR, depending on whether the lesions are located in the transcribed strand of active genes or elsewhere. To explore whether phosphorylation of γH2AX following UV irradiation is triggered by DNA intermediates generated by TCR, GGR or both, we used primary human fibroblasts derived from patients with specific defects in either or both of these pathways and analyzed γH2AX formation by flow cytometry. Whereas normal fibroblasts are proficient in both TCR and GGR, XP-C cells only have an operational TCR, CS-B cells only perform GGR and XP-A cells are defective in both pathways. In accordance with our western blot data presented in Figure 4, our flow cytometry data show that normal fibroblasts dramatically induce γH2AX formation when UV irradiation was delivered to cells that had been

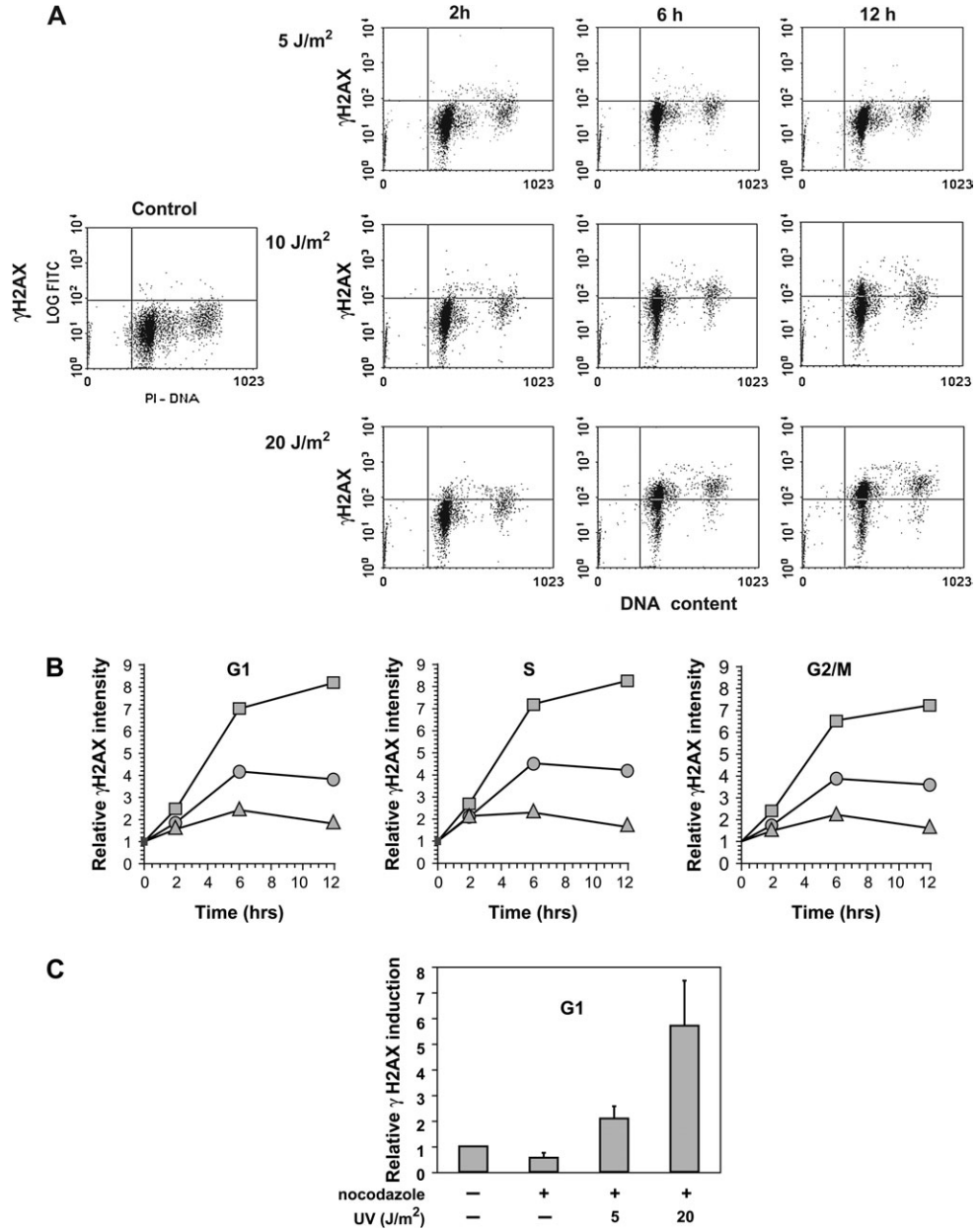

Fig. 3. Cell cycle-independent induction of H2AX phosphorylation after UVC. (**A**) Flow cytometry analysis showing γH2AX intensity as a function of DNA content (cell cycle position) in normal fibroblasts after different doses of UVC and different incubation times. Exponentially growing normal fibroblasts were exposed to 0 (control), 5, 10 or 20 J/m^{2}of UV light and allowed to recover for 2, 6 or 12 h at 37°C. Cells were then collected and analyzed by dual parameter flow cytometry. γH2AX intensity is represented on the Y-axis, while propidium iodide staining (DNA content) is plotted along the X-axis. (**B**) The data from (**A**) was plotted as mean γH2AX staining intensity relative to mock-irradiated (control) cells as a function of cell cycle position and time after irradiation. This experiment was repeated four times with similar results. The percentage of cells in the different phases of the cell cycle did not change significantly following post-UV incubations. The percentage of cells in S-phase for the different treatments were 7–12%. Symbols: triangles, 5 J/m^2; circles, 10 J/m^2; squares, 20 J/m^2. (**C**) Normal fibroblasts were grown to confluence and mock treated (control), treated with 50 ng/ml nocodazole for 6 h, irradiated with 5 or 20 J/m^{2}of UV light followed by 6 h recovery in medium containing nocodazole. Cells were harvested and analyzed for γH2AX content using flow cytometry as in (**A**). Only the G_1 population is represented in the graph and is the average of duplicate samples, with error bars representing the standard deviation.

pretreated with aphidicolin (Figure 5). Furthermore, γH2AX formation occurred in all phases of the cell cycle ruling out an exclusive role of DNA replication blockage in its induction. That the DNA repair intermediates were responsible for the γH2AX formation in NER-proficient cells was verified by the fact that addition of aphidicolin to UV-irradiated repair-deficient XP-A cells did not induce phosphorylation of H2AX. When these experiments were repeated using UV-irradiated GGR-deficient XP-C cells, we observed only a small induction of γH2AX when repair synthesis was inhibited by aphidicolin. In contrast, blockage of DNA repair synthesis in UV-irradiated TCR-deficient CS-B cells resulted in a dramatic increase in γH2AX formation. These results suggest that repair intermediates generated by GGR contribute much more prominently to γH2AX formation than do intermediates formed by TCR.

ATR kinase mediates H2AX phosphorylation upon accumulation of UV-induced DNA repair intermediates

The phosphorylation of H2AX in response to different types of stress has been shown to be dependent on various members of the PI(3)-like

kinases, such as ATM, ATR and DNA-PKcs. After UV light exposure, the ATR (20,21,26) and the Jun-c-terminal kinases(34) have been implicated in the induction of γH2AX. While it is generally accepted that the ATR kinase responds to replication stress, we wanted to learn if the replication-independent H2AX phosphorylation was also mediated by ATR as suggested by O'Driscoll *et al.* (22). In particular, the repair intermediates involved in NER could represent DNA ends or single-stranded regions, which could potentially stimulate ATM, ATR and/or DNA-PKcs activity. To study the potential roles of ATM, ATR and/or DNA-PKcs, we used wortmannin and caffeine. Wortmannin inhibits ATM and DNA-PKcs at doses <20 μM while at 100 μM wortmannin also inhibits ATR (35). Caffeine on the other hand inhibits ATM and ATR at lower doses (2 mM) while it also inhibits DNA-PKcs at higher doses (10 mM) (36).

We first explored the effects wortmannin may have on H2AX phosphorylation following accumulation of UV-induced DNA repair intermediates. As can be seen in Figure 6A and B, 5 J/m^2 induced a low level of γH2AX that was increased by pretreatment with aphidicolin to accumulate DNA repair intermediates. This level of γH2AX was reduced by treatment with 20 μM wortmannin and eliminated by treatment with 100 μM wortmannin. When these experiments were repeated with caffeine, we found that caffeine effectively eliminated γH2AX formation both when used at the 2 and 10 mM concentration (Figure 6C and D). These results are consistent with a model where the ATR kinase phosphorylates H2AX in response to accumulation of DNA repair intermediates.

To verify that the ATR kinase is responsible for phosphorylating H2AX following accumulation of DNA repair intermediates, we took a genetic approach using cell lines with specific defects in either ATM or ATR. Cells derived from certain patients with the Seckel syndrome have a severely attenuated expression of the ATR kinase (22), whereas cells derived from patients with ataxia telengiactasia express no functional ATM kinase. Using these cell lines, we found that H2AX phosphorylation induced by accumulation of DNA repair intermediates was reduced somewhat in UV-irradiated AT cells as compared with UV-irradiated control cells (6) (Figure 6E and F). However, induction of γH2AX was clearly reduced in UV-irradiated Seckel syndrome cells pretreated with aphidicholin to accumulate DNA repair intermediates. Taken together, our studies using wortmannin and caffeine and the studies using the ATM- and ATR-deficient cell lines suggest that the ATR kinase is activated by DNA repair intermediates, leading to the phosphorylation of H2AX.

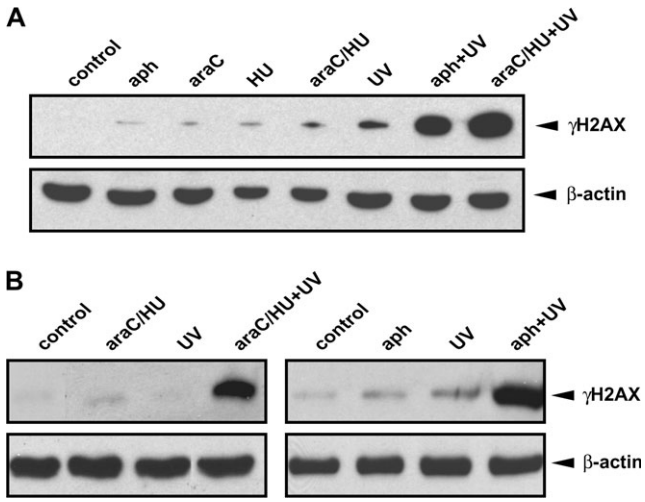

Fig. 4. The role of DNA repair intermediates in the induction of γH2AX. (**A**) Western blot analysis showing γH2AX formation in exponentially growing normal fibroblasts after UV irradiation and inhibition of DNA repair synthesis. Cells were pretreated for 30 min with 1 μg/ml aphidicolin (aph), 10 μM araC, 2 mM HU or the combination of araC and HU (araC/HU). The cells were then mock irradiated or exposed to 5 J/m^{2}of UV light and allowed to recover for 6 h in the presence of the inhibitors. β-actin is shown as a loading control. (**B**) Normal fibroblasts were serum starved for 72 h to arrest them in the G_0/G_1phase of the cell cycle and then treated with araC/HU and UV (left panels) or aphidicolin and UV (right panels) similar to (**A**).

Discussion

In this study, we investigated the mechanisms by which γH2AX is induced following UV irradiation. It is thought that bulky adducts induced by UV light cause replication fork stalling and eventual fork collapse, leading to the formation of DSB which are known to trigger γH2AX formation (24,37,38). Furthermore, it has been proposed that unprocessed DNA lesions are not sufficient for H2AX phosphorylation following UV irradiation (21). Our study and studies of others (24–27) demonstrate that H2AX phosphorylation occurs in all phases of the cell cycle and, therefore, other mechanisms in addition to S-phase-dependent DSB formation must contribute to γH2AX formation following UV irradiation.

One possible trigger for the replication-independent induction of H2AX phosphorylation following UV irradiation could be the generation of DNA repair-induced intermediates (22,27). As UV-induced lesions are removed from DNA, stretches of ∼30 nucleotides are excised leaving gaps in the DNA template. These gaps are filled in by DNA polymerases followed by ligation by DNA ligases (18). By using the DNA polymerase alpha inhibitor aphidicolin or the nucleotide pool synthesis inhibitor HU together with the nucleotide analogue araC to accumulate DNA repair intermediates (30–33), we found that UV-induced induction of H2AX phosphorylation was dramatically increased in NER-proficient human fibroblasts but not in

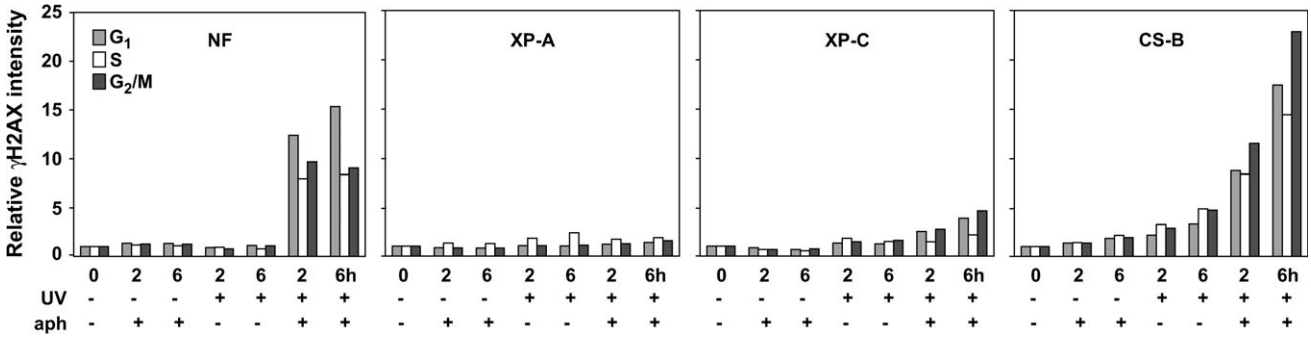

Fig. 5. Accumulation of NER intermediates results in enhanced H2AX phosphorylation in all phases of the cell cycle. Normal fibroblasts, XP-A, XP-C or CS-B fibroblasts were mock treated (control), treated with 1 μg/ml aphidicolin (aph), exposed to 5 J/m^2(UV) or pretreated for 30 min with aphidicolin followed by UV irradiation and incubation in medium containing aphidicolin. Two or 6 h later, cells were harvested and analyzed for γH2AX staining intensity by flow cytometry. The mean intensity of γH2AX in each phase of the cell cycle was analyzed separately and normalized to that of the control sample.

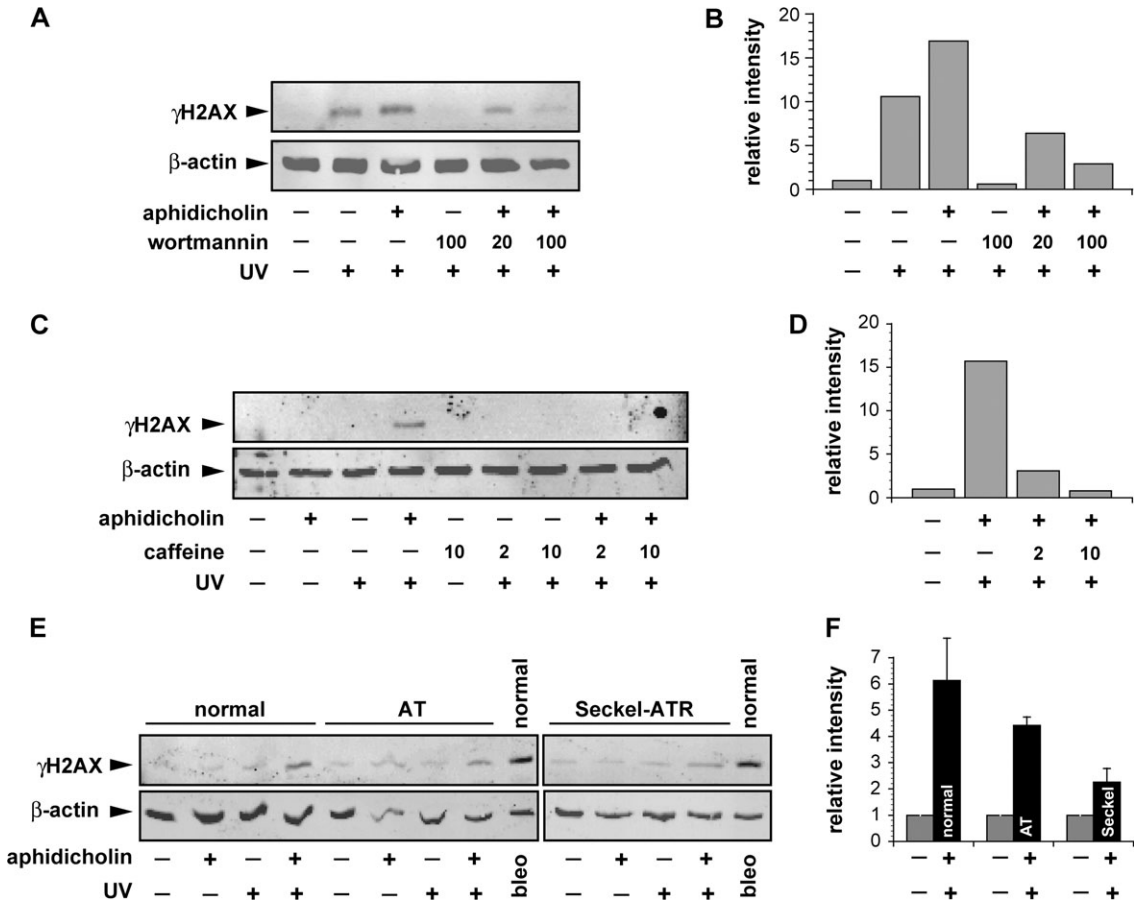

Fig. 6. ATR mediates H2AX phosphorylation triggered by the accumulation of repair intermediates. (**A**) Serum-starved normal fibroblasts pretreated with aphidicolin with or without wortmannin (20 or 100 μM) for 30 min prior to 5 J/m^{2}UV irradiation and allowed to recover for 2 h in the presence of respective drugs. Western blot shows γH2AX (top panel) and β-actin (bottom panel). (**B**) Bands of γH2AX were quantified using NIH Image software and were plotted relative to untreated control samples. (**C**) Caffeine (2 or 10 mM) was used instead of wortmannin in a similar experiment to (**A**). (**D**) Bands of γH2AX were quantified using NIH Image software and were plotted relative to untreated control samples (**E**) Normal, AT or Seckel-ATR lymphoblasts were treated with aphidicolin, UV light or the combination of aphidicolin and UV light as before and analyzed for the induction of γH2AX. Bleomycin (5 μg/ml for 2 h) treated cells were used as positive control and as an indicator of equal exposure of the blots and β-actin was used as a loading control. (**F**) Bands of γH2AX were quantified using NIH Image software and were plotted relative to untreated control samples. The bars represent the mean value from two independent experiments ±SEM.

NER-deficient XP-A fibroblasts (Figure 5). Furthermore, inhibition of DNA repair synthesis in UV irradiated CS-B cells resulted in significantly higher induction of γH2AX than in XP-C cells, suggesting that accumulated GGR intermediates contribute much more to the induction of γH2AX than accumulated TCR intermediates.

The mechanism by which DNA repair intermediates trigger γH2AX formation is not clear but the fact that DNA ends are generated during the incision step of NER suggests that DNA breaks may be the trigger for the induced H2AX phosphorylation. Moreover, we cannot rule out that the inhibition of the rejoining step of repair by the use of the different DNA polymerase inhibitors results in the induction of DSB, which in turn would induce H2AX phosphorylation in an ATM-dependent manner (9,39). However, we found only a minor role for ATM in the induction of γH2AX following accumulation of DNA repair intermediates, as AT cells showed only a slightly reduced induction of H2AX phosphorylation compared with control cells (Figure 6E and F). In contrast, inhibition of ATR function or deficient expression of ATR resulted in diminished levels of γH2AX, following accumulation of DNA repair intermediates. Although the phosphorylation of H2AX in ATR-deficient Seckel syndrome cells is not absent, it is reduced. It is possible that the low level of ATR that is still expressed in these cells is sufficient to phosphorylate H2AX to some extent. Alternatively, other Wortmannin- and caffeine-sensitive kinases may, in addition to ATR, contribute to the UV-induced phosphorylation of H2AX. Our findings demonstrating that ATR kinase participates in the induction of H2AX phosphorylation following UV irradiation is in concurrence with previous studies (22,26,27).

While the contribution of DSB to the induction of γH2AX is well established, it is now increasingly evident that other types of lesions or cellular processes can induce γH2AX as well. Constitutive H2AX phosphorylation in unstressed human cells has been reported which is unrelated to DNA damage and is dependent on the ATM kinase (40,41). This constitutive ATM activation and H2AX phosphorylation have been suggested to be the result of oxidative stress (42). Additionally, stresses that do not directly induce DSB have been shown to cause γH2AX formation [compiled in (43)]. The results from this study suggest that the early replication-independent induction of γH2AX following UV irradiation is triggered by DNA structures generated during the processing of UV-induced lesions and that this phosphorylation is mediated predominantly by the ATR kinase. Future studies are aimed at elucidating in more detail the precise roles of ATR and γH2AX in the DNA damage response following UV irradiation.

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