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Summary

Human histone H2AX is rapidly phosphorylated on serine 139 in response to DNA double-strand breaks and plays a crucial role in tethering the factors involved in DNA repair and damage signaling. Replication stress caused by hydroxyurea or UV also initiates H2AX phosphorylation in S-phase cells, although UV-induced H2AX phosphorylation in non-cycling cells has recently been observed. Here we study the UV-induced H2AX phosphorylation in human primary fibroblasts under growth-arrested conditions. This reaction absolutely depends on nucleotide excision repair (NER) and is mechanistically distinct from the replication stress-induced phosphorylation. The treatment of cytosine-β-D-arabinofuranoside strikingly enhances the NER-dependent H2AX phosphorylation and induces the accumulation of replication protein A (RPA) and ATR-interacting protein (ATRIP) at locally UV-damaged subnuclear regions. Consistently, the phosphorylation appears to be mainly mediated by ataxia-telangiectasia mutated and Rad3-related (ATR), although Chk1 (Ser345) is not phosphorylated by the activated ATR. The cellular levels of DNA polymerases δ and ϵ and proliferating cell nuclear antigen are markedly reduced in quiescent cells. We propose a model that perturbed gap-filling synthesis following dual incision in NER generates single-strand DNA gaps and hence initiates H2AX phosphorylation by ATR with the aid of RPA and ATRIP.

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Key words: Nucleotide excision repair, Histone H2AX, ATM and Rad3-related (ATR)

Introduction

Histone H2AX is a variant form of the core histone H2A and has a conserved SQ motif known to be a target for phosphorylation by phosphoinositide-3-kinase-related protein kinases (PIKKs) (West and Bonner, 1980). In humans, the serine 139 residue within this motif is phosphorylated in response to DNA double-strand breaks (DSB), and ATM (ataxia-telangiectasia mutated) is the principal kinase in this reaction (Fernandez-Capetillo et al., 2004), although DNA-dependent protein kinase (DNA-PK) functions redundantly to phosphorylate H2AX following DSB formation (Stiff et al., 2004). This phosphorylation takes place within a minute after exposure to ionizing radiation (IR) (Rogakou et al., 1998) and can also be observed during various cellular processes, including V(D)J recombination, meiotic recombination and apoptosis (Fernandez-Capetillo et al., 2004). These phosphorylation events are easily detected as nuclear foci by specific antibodies to the phosphorylated form of H2AX (γ-H2AX), and the foci formation has been extensively used as a marker of DSB formation. IR-induced foci of γ-H2AX have been reported to colocalize with those of BRCA1, Rad50, Nbs1, Rad51, p53 binding protein 1 (53BP1) and MDC1 (Paull et al., 2000; Schultz et al., 2000; Rappold et al., 2001; Goldberg et al., 2003; Lou et al., 2003; Stewart et al., 2003), suggesting a possible role of γ-H2AX in retaining various factors involved in DNA repair and damage signaling at damaged sites of the genome.

Chen and colleagues reported that hydroxyurea (HU) and UV irradiation also induce γ-H2AX formation only in S-phase cells and that γ-H2AX foci colocalize with proliferating cell nuclear antigen (PCNA), suggesting that H2AX participates in the surveillance of DNA replication (Ward and Chen, 2001). This replication stress-induced H2AX phosphorylation seems to depend on ATR (ATM and Rad3-related), but not ATM, and seems to colocalize with 53BP1 and BRCA1. The same group further reported that single-stranded DNA (ssDNA) regions generated by replication arrest initiate the ATR-mediated phosphorylation of H2AX, which in turn stabilizes ATR at the sites of arrested replication forks (Ward et al., 2004). Recently, another group showed that UV-induced cyclobutane pyrimidine dimer (CPD) is a major source of DNA breaks following UV, based on transcriptome analysis using

fibroblasts derived from transgenic mice expressing marsupial CPD-specific photolyase (Garinis et al., 2005). During DNA replication, unrepaired CPD can be converted into DSB and provokes the time-dependent accumulation of γ-H2AX, 53BP1 and Rad51 foci, which coincides with accumulation of cells in S phase.

The model that UV-induced H2AX phosphorylation is mediated by replication stress has been challenged by the observation that UV-induced H2AX phosphorylation occurs in non-replicating human cells under low-serum conditions (O'Driscoll et al., 2003). This phosphorylation is undetectable in fibroblasts derived from an individual affected with xeroderma pigmentosum (XP), a hereditary disorder characterized by a hyper-sensitivity to UV light, a high incidence of skin cancer in sun-exposed areas and a defective nucleotide excision repair (NER). The NER is a major repair system for removing a variety of DNA lesions, including UV-induced CPD and (6-4) photoproduct (6-4PP) as well as chemical-induced bulky base adducts (Friedberg et al., 2006). The biochemical process of NER has been extensively studied and reconstituted in vitro with purified proteins (Mu et al., 1995; Aboussekhra et al., 1995). The basic NER reaction can be largely divided into two stages by a dual-incision step. The first stage (pre-incision) is initiated by damage recognition and subsequent local unwinding around a damaged site, which can be achieved by XPC-HR23B, XPA, replication protein A (RPA) and TFIIH. The resultant bubble-like intermediate allows two structure-specific endonucleases, XPG and XPF-ERCC1, to make a dual incision on both sides of the lesion. A post-incision stage consists of gap-filling synthesis and rejoining by replication factors including replication factor C (RFC), PCNA, DNA polymerase (Pol) δ and/or ϵ and DNA ligase I. It was clearly shown that ssDNA created during NER are not sufficient to provoke ATR activation in cycling cells (Ward et al., 2004), and the link between NER and UV-induced H2AX phosphorylation is still unknown.

In this study, we show that in quiescent human cells the NER process can be perturbed at a gap-filling step because of a lack of replication factors, and the resultant ssDNA-gap intermediates or their processed derivatives initiate the phosphorylation of histone H2AX in an ATR-dependent manner.

Results

UV-induced H2AX phosphorylation occurs independently in quiescent cells and cycling S-phase cells

Normal human fibroblast MSU-2 cells were cultured under two different conditions, actively growing in complete medium containing 10% fetal bovine serum (FBS) or growth-arrested by contact inhibition followed by serum starvation (0.5% FBS), exposed to UV-C and tested for γ-H2AX formation using immunostaining. For the asynchronously growing population, S-phase cells were pulse-labeled with bromodeoxyuridine (BrdU) just before fixation and detected by anti-BrdU antibody. As shown in Fig. 1A, S-phase cells showed strong signals of γ-H2AX, whereas BrdU-negative non-S-phase cells did not, consistent with an earlier report (Ward and Chen, 2001). In the growth-arrested (quiescent) cell population, however, almost all cells exhibited a significant γ-H2AX formation (Fig. 1A), as previously observed (O'Driscoll et al., 2003). We decided to analyze the two types of UV-induced H2AX phosphorylation more quantitatively using flow cytometry (Fig. 1B). Actively growing cells were divided into three subpopulations (G1, S and G2/M) based on the staining with propidium iodide (PI) and anti-BrdU antibody. Following UV irradiation, the level of γ-H2AX signal increased most profoundly in S-phase cells, although G1- and G2/M-phase cells also showed a small but reproducible increase in the level of γ-H2AX. In quiescent cells, UV exposure caused a moderate shift of the whole subpopulation, consistent with the results from immunostaining.

In order to define the relationship between the two phosphorylation events, quiescent cells were released by subculturing at lower density with complete medium and exposed to UV at 12, 15 or 18 hours after the release. The signal intensity as well as the frequency of γ-H2AX-positive cells gradually decreased, with the exception of strong signals seen in BrdU-positive S-phase cells at 18 hours (Fig. 1C). These results clearly indicate that two independent pathways of H2AX phosphorylation can be activated by UV: one is specifically observed in cycling S-phase cells (replication-dependent) and another in serum-starved quiescent cells (replication-independent).

UV-induced H2AX phosphorylation in quiescent cells occurs at damaged DNA sites and requires NER activity

In order to determine whether the replication-independent γ-H2AX formation induced by UV is spatially restricted to damaged DNA sites, we took advantage of a micropore UV-irradiation technique that enables us to locally induce UV lesions in the nucleus (Volker et al., 2001; Katsumi et al., 2001). Quiescent MSU-2 cells were exposed to UV through an isopore membrane filter, cultured for various periods and co-immunostained with the antibodies against γ-H2AX and CPD (Fig. 2A). The signals of γ-H2AX completely colocalized with CPD signals, demonstrating that H2AX phosphorylation occurs at damaged subnuclear regions. The signal intensity of γ-H2AX was constantly high at 1-4 hour(s) and decreased at 6 hours, although weak signals could be detected at 15 minutes (data not shown). The time course of γ-H2AX formation appears to correlate with repair kinetics showing fast removal of 6-4PP within 4 hours and slow removal of CPD (Fig. 2).

To examine whether NER is required for the UV-induced H2AX phosphorylation in quiescent cells, XP2BI (XP-G) cells lacking one of the endonucleases involved in a dual-incision step of NER were exposed to UV. The repair abilities of CPD and 6-4PP were almost completely abrogated in XP2BI cells (see supplementary material Fig. S1) and immunofluorescence signals of γ-H2AX were strikingly diminished under growth-arrested conditions, although S-phase-specific H2AX phosphorylation occurred as efficiently as that seen in normal cells (Fig. 3A,B). Flow cytometric analyses confirmed the results obtained by immunostaining and further revealed that neither XP12BE (XP-A) nor XP1CTA (XP-C) cells with an NER-defect (see supplementary material Fig. S1) show UV-induced γ-H2AX formation, although another NER-proficient cell strain, TIG-120, exhibited a comparable level of γ-H2AX signal with MSU-2 cells (Fig. 3C). Moreover, we asked whether the transfection of wild-type XPA cDNA recovers the H2AX phosphorylation in quiescent XP-A cells following UV irradiation. XP3OS/T-n cells immortalized by the introduction

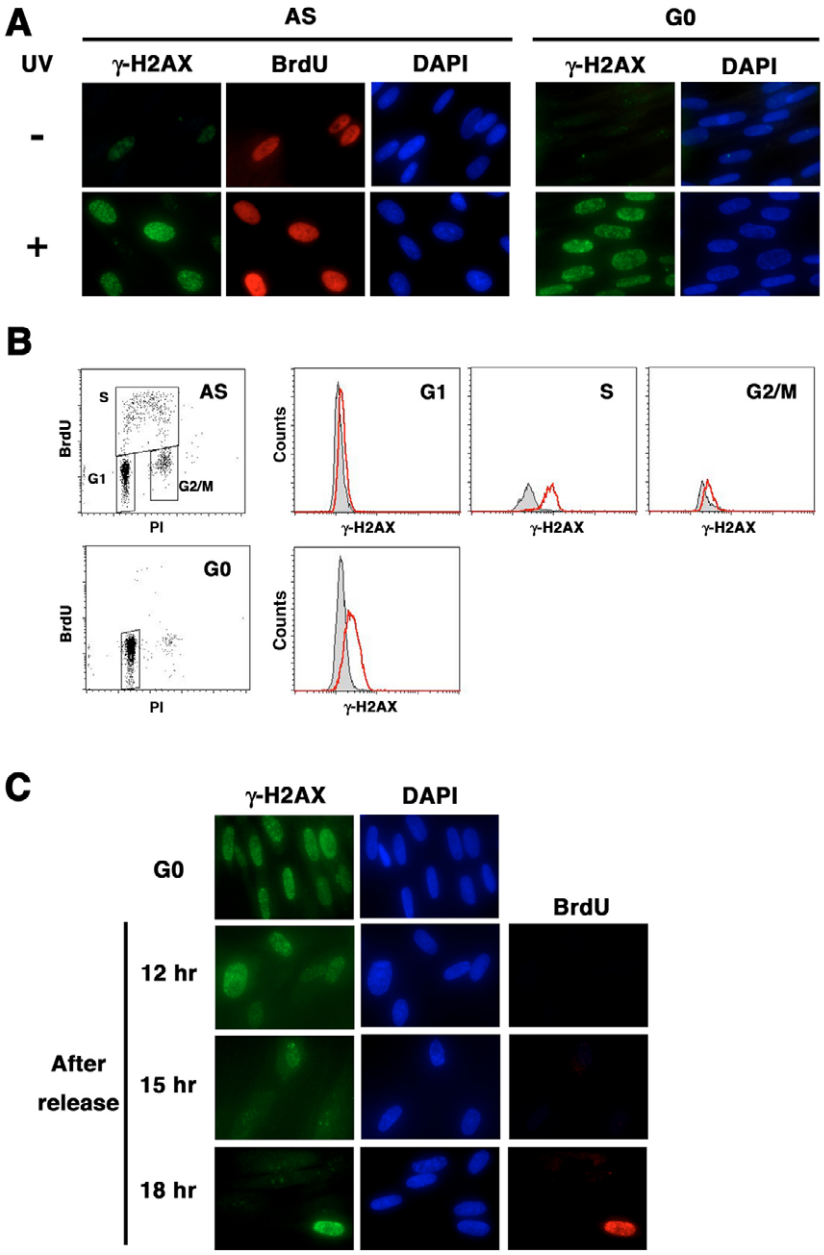

Fig. 1. UV-induced H2AX phosphorylation occurs independently in quiescent cells and cycling S-phase cells. (A) MSU-2 cells growing asynchronously (AS) or growth-arrested by contact inhibition and serum starvation (G0) were irradiated with 10 J/m^2 UV, incubated for 4 hours and treated with 50 μM BrdU for 15 minutes before fixation. Cells were stained with anti-γ-H2AX and anti-BrdU antibodies and DAPI was used for nuclear counterstaining. Note that the BrdU-labeling index was less than 1% in the growth-arrested population. (B) MSU-2 cells (AS or G0) were irradiated with UV (black line, 0 J/m^2; red line, 10 J/m^2), incubated for 1 hour and treated with 50 μM BrdU for 30 minutes before fixation. Cells were stained with anti-γ-H2AX and anti-BrdU antibodies, and PI staining was performed to measure cellular DNA content. For the asynchronous population, cells were divided into three subpopulations (G1, 65%; S, 19%; G2/M, 14%) based on the staining with PI and anti-BrdU antibody, and histograms of γ-H2AX intensity in each subpopulation are shown in the upper panels. For quiescent cells, a BrdU-negative 2N subpopulation (92%) was analyzed and shown as a histogram of γ-H2AX in the lower panel. (C) Growth-arrested MSU-2 cells were released by subculturing at lower density under normal serum conditions and irradiated with 10 J/m^2 UV at 12, 15 or 18 hours after the release. The cells were incubated for 30 minutes and processed for immunostaining as described in A.

of the human telomerase reverse transcriptase (hTERT) gene were used for this experiment and this cell line also exhibited neither NER activity nor UV-induced H2AX phosphorylation under growth-arrested conditions (see supplementary material Fig. S2A,B). The ectopic expression of wild-type XPA complemented the impairment of XP3OS/T-n in NER activity (see supplementary material Fig. S2C) as well as UV-induced H2AX phosphorylation in a quiescent state (Fig. 3D). Together, these results clearly indicate that UV-induced γ-H2AX formation in quiescent cells depends on NER activity.

The implication of NER in this signaling pathway prompted us to test whether other DNA-damaging agents can induce the phosphorylation of H2AX in quiescent cells. MSU-2 and XP2BI cells were treated with N-acetoxy-2-acetylaminofluorene (NA-AAF), which produces bulky base adduct, a well-known substrate for NER, and tested for H2AX phosphorylation. As expected, NA-AAF induced H2AX phosphorylation in growth-arrested MSU-2 cells but not XP2BI cells (Fig. 3E), suggesting that not only UV lesions but also other substrates for NER are able to induce the H2AX phosphorylation in quiescent cells. It should be noted that NA-AAF-induced H2AX phosphorylation can be observed in asynchronously growing XP2BI cells and that this reaction is again S-phase-specific (Fig. 3E).

The NER-dependent H2AX phosphorylation is possibly caused by inefficient gap-filling synthesis following dual incision

What is a trigger for the NER-dependent H2AX phosphorylation? We suspected that ssDNA gaps generated by dual incision might be a candidate for the trigger, since all XP cell strains used here have a defect in a dual-incision step of NER. In order to test this possibility, the quiescent human cells were globally exposed to UV and incubated in the presence of cytosine-β-D-arabinofuranoside (Ara-C) to block a gap-filling reaction. The level of γ-H2AX formation was remarkably enhanced by Ara-C in MSU-2 but not XP2BI cells (Fig. 4A), and the same effect was also observed in locally UV-irradiated cells (Fig. 4B). By contrast, Ara-C treatment showed no effects on γ-H2AX formation in MSU-2 cells treated with etoposide producing DSB (Fig. 4C), indicating that perturbation of a gap-filling step of NER induces H2AX phosphorylation, possibly by generating ssDNA-gap intermediates.

The next question is why in quiescent cells, but not cycling cells, the gap-filling reaction can be perturbed even without Ara-C. We measured the cellular levels of RPA (RPA2 p34 subunit),

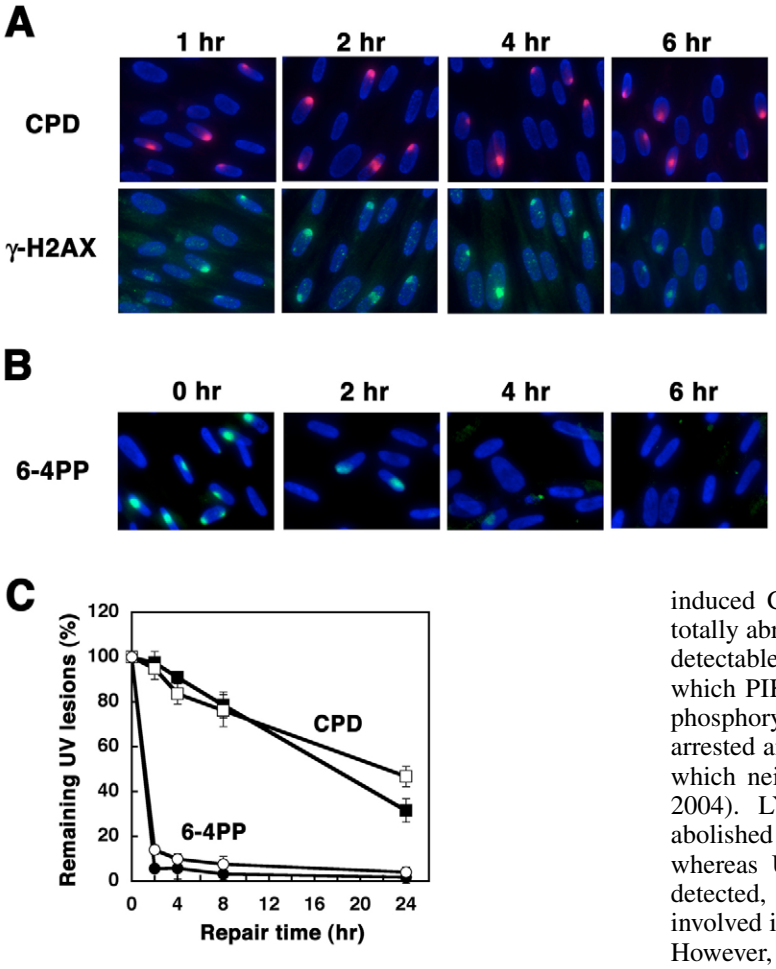

Fig. 2. UV-induced H2AX phosphorylation in quiescent cells occurs at damaged DNA sites and correlates with repair kinetics of UV lesions. (A,B) Growth-arrested MSU-2 cells were locally irradiated with 100 J/m^2 UV through an isopore membrane filter and incubated for various periods. Cells were fixed and stained with anti-γ-H2AX and anti-CPD antibodies (A), or anti-6-4PP antibody alone (B). (C) MSU-2 cells growing asynchronously (closed symbols) or growth-arrested (open symbols) were irradiated with 10 J/m^2 UV and incubated for the indicated periods. Genomic DNA was isolated and the amounts of CPD and 6-4PP were determined using an enzyme-linked immunosorbent assay. Each point represents the mean of three experiments and bars indicate the s.d.

PCNA and the catalytic subunit (CS) of Pol δ or ϵ, which had been shown to function in the gap-filling reaction using an in vitro system (Coverley et al., 1991; Nichols and Sancar, 1992; Shivji et al., 1992; Zeng et al., 1994a; Shivji et al., 1995; Aboussekhra et al., 1995). Immunoblot analysis revealed that the amounts of Pol δ (125-kDa CS), Pol ϵ (261-kDa CS) and PCNA, but not RPA2, were markedly reduced in quiescent cells compared with the asynchronously growing population (Fig. 5A). By contrast, the cellular level of XPA required for the pre-incision stage of NER was relatively steady, consistent with the comparable repair kinetics of UV lesions between the two culturing conditions (Fig. 2C). Furthermore, the levels of Pol δ (125-kDa CS) and Pol ϵ (261-kDa CS) gradually increased after release from the growth-arrested state (Fig. 5B), and the recovery pattern corresponded well with the time-dependent decrease of γ-H2AX signals, as shown in Fig. 1C. Taken together, these results suggest that the limited amounts of replication factors such as Pol δ (125-kDa CS), Pol ϵ (261-kDa CS) and/or PCNA in quiescent cells might cause inefficient gap-filling synthesis, and hence prolonged existence of ssDNA-gap intermediates.

ATR is the principal kinase for the NER-dependent H2AX phosphorylation

The NER-dependent H2AX phosphorylation was sensitive to caffeine, an inhibitor for PIKKs, under the conditions that UV-induced Chk1 phosphorylation in cycling MSU-2 cells was totally abrogated, although weak signals of γ-H2AX were still detectable (see supplementary material Fig. S3). We asked which PIKK (ATM, DNA-PK or ATR) is responsible for the phosphorylation. ATM-deficient AT2KY cells were growth-arrested and treated with LY294002 to obtain the condition in which neither ATM nor DNA-PK is functional (Stiff et al., 2004). LY294002 treatment to AT2KY cells completely abolished the H2AX phosphorylation induced by etoposide, whereas UV-induced H2AX phosphorylation was evidently detected, indicating that ATM and DNA-PK are not mainly involved in this reaction (see supplementary material Fig. S4). However, GM18366 cells derived from an ATR-Seckel syndrome patient with only a marginal level of functional ATR showed a largely affected phenotype of UV-induced H2AX phosphorylation under growth-arrested conditions (Fig. 6A), although etoposide-induced phosphorylation appeared to be normal (Fig. 6B). These results strongly suggest that ATR is the principal kinase for the NER-dependent H2AX phosphorylation.

RPA has been shown to play a crucial role in the recruitment of ATR and ATR-interacting protein (ATRIP) complex to ssDNA regions (Zou and Elledge, 2003). We tried to detect those factors accumulating at locally damaged sites in the nucleus, but failed probably because of insufficient sensitivity of the antibodies used. However, the treatment of Ara-C allowed us to detect local accumulation of RPA as well as ATRIP in quiescent cells subjected to micropore UV irradiation (Fig. 6C,D), correlating with the enhanced H2AX phosphorylation by Ara-C treatment (Fig. 4). Again, the stimulatory effect of Ara-C on the accumulation of RPA and ATRIP was not observed in NER-deficient XP2BI cells (Fig. 6C,D). These data lead us to speculate that RPA coats the ssDNA-gap intermediates created by perturbed gap-filling synthesis, possibly facilitating the recruitment of ATRIP with ATR.

The NER-dependent H2AX phosphorylation coincides with 53BP1 accumulation at damaged sites but not with Chk1-Ser345 phosphorylation

We next asked whether the NER-mediated ATR activation in

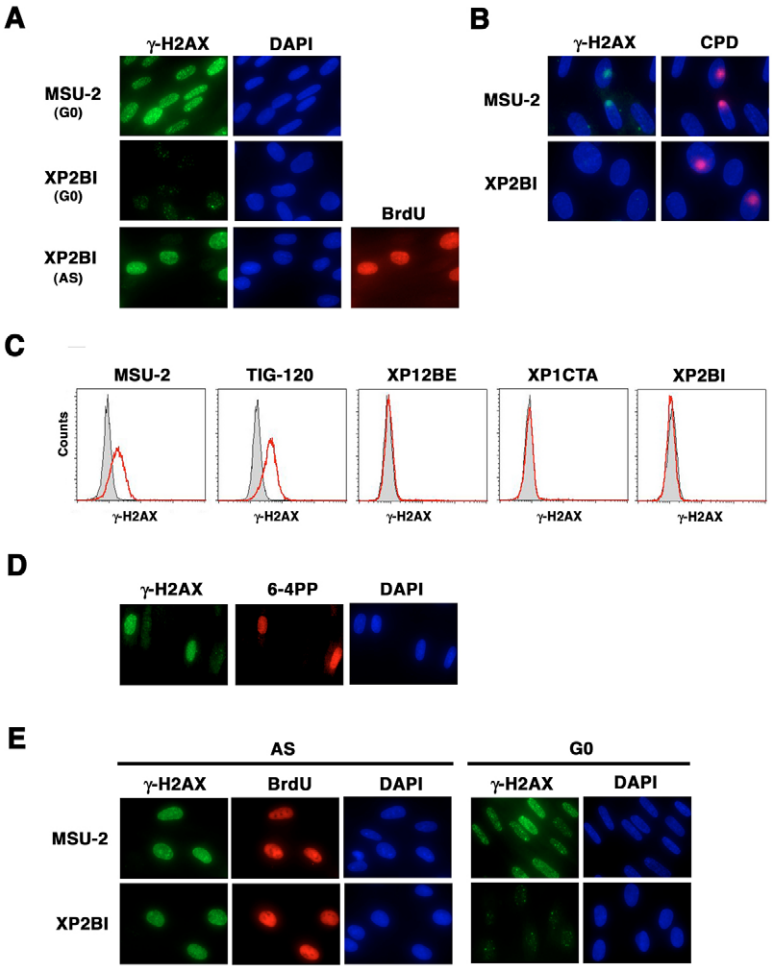

Fig. 3. Nucleotide excision repair is required for the UV-induced H2AX phosphorylation in quiescent cells. (A) MSU-2 or XP2BI cells (AS or G0) were irradiated with 10 J/m^2 UV and incubated for 4 hours. 50 μM BrdU was included in the asynchronous population for 15 minutes before fixation and cells were costained with anti-γ-H2AX and anti-BrdU antibodies. (B) MSU-2 or XP2BI cells under growth-arrested conditions were locally irradiated with 40 J/m^2 UV and incubated for 4 hours. Cells were fixed and double-stained with anti-γ-H2AX and anti-CPD antibodies. (C) MSU-2, TIG-120, XP12BE, XP1CTA and XP2BI cells growth-arrested by contact inhibition and serum starvation were irradiated with no UV (black lines) or 10 J/m^2 UV (red lines) and incubated for 1 hour before fixation. Cells were stained with anti-γ-H2AX and PI and analyzed by flow cytometry. (D) XP3OS/T-n cells were transfected with pCMV-Myc-XPA plasmid using TransFectin lipid reagent (Bio-Rad) and incubated for 2 days. The cells were growth-arrested by serum starvation for 4 days and exposed to 20 J/m^2 UV. After 3 hours of incubation, cells were fixed and double-stained with anit-γ-H2AX and anti-6-4PP antibodies. (E) MSU-2 or XP2BI cells (AS or G0) were treated with 2 μM NA-AAF for 30 minutes and incubated for another 30 minutes after medium change. BrdU was used for labeling S-phase cells in the AS population, and γ-H2AX and BrdU were detected as described in A.

quiescent cells also causes the phosphorylation of Chk1, which is a well-known target for ATR in response to HU or UV treatment. NER-proficient MSU-2 cells were exposed to UV and tested for Chk1 phosphorylation using a phospho-Ser345-specific antibody. As shown in Fig. 7A, UV-induced phosphorylation of Chk1 on Ser345 was observed in asynchronously growing cells but not in quiescent cells, indicating that the NER-mediated ATR activation targets H2AX Ser139 but not Chk1 Ser345. It should also be noted that the cellular level of Chk1 itself is significantly reduced in quiescent cells, consistent with a previous report (Chini and Chen, 2003).

We next tried to identify the factors that accumulate at damaged DNA sites following local UV irradiation under quiescent conditions. We carefully performed these experiments using a low dose of UV to avoid inducing DSB and found that 53BP1 accumulates at locally UV-damaged sites (Fig. 7B,C). Similarly, the accumulation of 53BP1 was enhanced by Ara-C and compromised in NER-deficient XP1CTA (XP-C) cells. These findings suggest that 53BP1 might be implicated in the NER-dependent H2AX phosphorylation upstream or downstream.

Discussion

In this study, we have examined UV-induced H2AX phosphorylation independent of DNA replication but dependent on NER, which is predominantly detected under growth-arrested conditions. We and others have failed to detect UV-induced γ-H2AX foci in synchronously cycling G1 cells using immunostaining (Fig. 1C) (Ward and Chen, 2001; Ward et al., 2004). However, flow cytometric analysis revealed weak γ-H2AX signals in G1-phase (also G2/M-phase) cells of the asynchronous population following UV (Fig. 1B). While this paper was under consideration, Cleaver and co-workers reported similar observations with a higher UV dose (20 J/m^2), and showed that the UV-induced H2AX phosphorylation within the G1 phase depends on NER (Marti et al., 2006). Interestingly, we could observe γ-H2AX foci in cycling non-S-phase cells when treated with Ara-C after UV irradiation (data not shown). These results suggest that irrespective of culturing condition and cell cycle phase, perturbation of gap-filling synthesis can induce H2AX phosphorylation and that this event indeed happens even in cycling cells, although with much less frequency compared with quiescent cells. In conclusion, we propose a model that in quiescent cells, uncoupling of dual incision and gap-filling takes place during NER because of extremely low levels of replication factors and the resultant ssDNA-gap intermediates cause H2AX phosphorylation (Fig. 8).

Studies using specific inhibitors or antibodies to various DNA polymerases revealed that Pol δ and/or Pol ϵ, but neither Pol α nor Pol β, are responsible for the gap-filling reaction

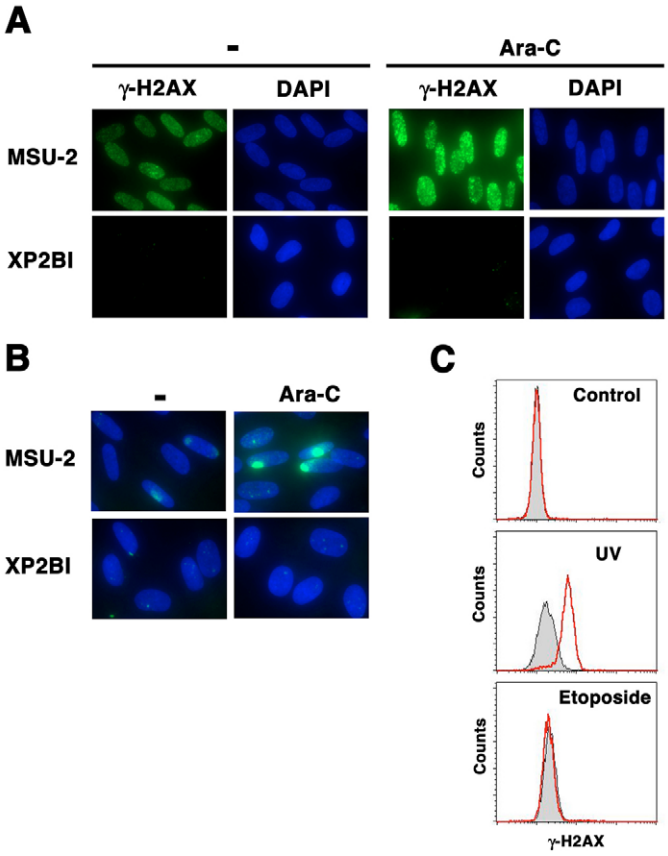

Fig. 4. Perturbation of repair replication in NER enhances H2AX phosphorylation. (A,B) MSU-2 or XP2BI cells under growth-arrested conditions were exposed to 10 J/m^2 UV globally (A) or locally (B), incubated for 1 hour in the absence or presence of 100 μM Ara-C before fixation and stained with anti-γ-H2AX antibody. (C) Growth-arrested MSU-2 cells were exposed to 10 J/m^2 UV or 15 μg/mL etoposide and incubated for 1 hour in the presence (red lines) or absence (black lines) of Ara-C before fixation. Cells were stained with anti-γ-H2AX antibody and PI and analyzed by flow cytometry.

following dual incision (Wood and Shivji, 1997). PCNA, a processivity factor for Pol δ and Pol ϵ, has also been required for in vitro NER reaction with cell-free extracts (Nichols and Sancar, 1992; Shivji et al., 1992). Indeed, the in vitro gap-filling reaction was reconstituted with purified proteins of PCNA, RFC, RPA and either Pol δ or Pol ϵ (Aboussekhra et al., 1995; Shivji et al., 1995). These factors are originally known as components of the machinery for semi-conservative replication of chromosomal DNA, and the steady-state levels of PCNA, Pol δ (125-kDa CS) and Pol ϵ (261-kDa CS) are cell cycle-dependent and most abundant at the G1/S boundary (Morris and Mathews, 1989; Zeng et al., 1994b; Tuusa et al., 1995). Moreover, the expression levels of Pol δ (125-kDa CS) and Pol ϵ (261-kDa CS) are marginal in serum-starved quiescent cells and are dramatically increased by serum stimulation (Tuusa et al., 1995; Yang et al., 1992), consistent with our data (Fig. 5B).

The NER-dependent H2AX phosphorylation seems to be mediated mainly by ATR but not ATM and DNA-PK (Fig. 6A,B and see supplementary material Figs S3, S4), consistent with the recent observation that the cells derived from an ATR-Seckel syndrome patient harboring a splicing mutation affecting expression of ATR display a defective UV-induced H2AX phosphorylation under low-serum conditions as well as asynchronously growing conditions (O'Driscoll et al., 2003). It has been suggested that ATR is recruited to RPA-covered ssDNA with the aid of its small partner ATRIP (Zou and

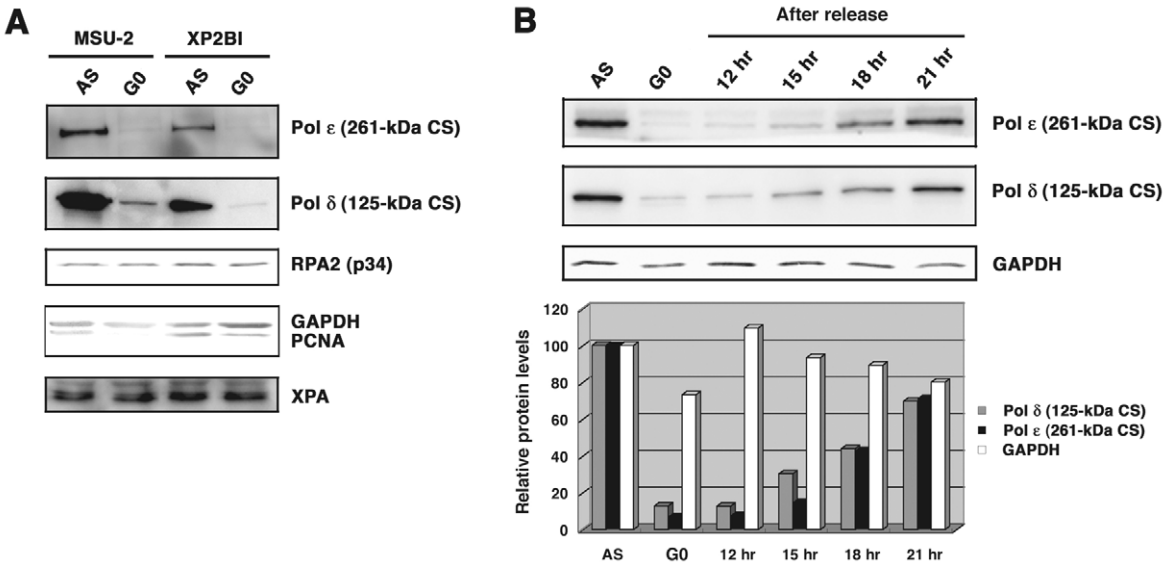

Fig. 5. Cellular levels of repair replication factors are markedly reduced in quiescent cells. (A) MSU-2 or XP2BI cells (AS or G0) were lysed and cellular levels of the indicated proteins were analyzed by immunoblotting with specific antibodies. (B) Growth-arrested MSU-2 cells were released as described in the legend of Fig. 1C. Cell lysates were prepared at the indicated time points and used for immunoblotting with anti-DNA Pol δ (125-kDa CS) or anti-DNA Pol ϵ (261-kDa CS) antibodies. The quantitative data are shown in the bottom panel and represent the relative protein levels (%) to the AS sample.

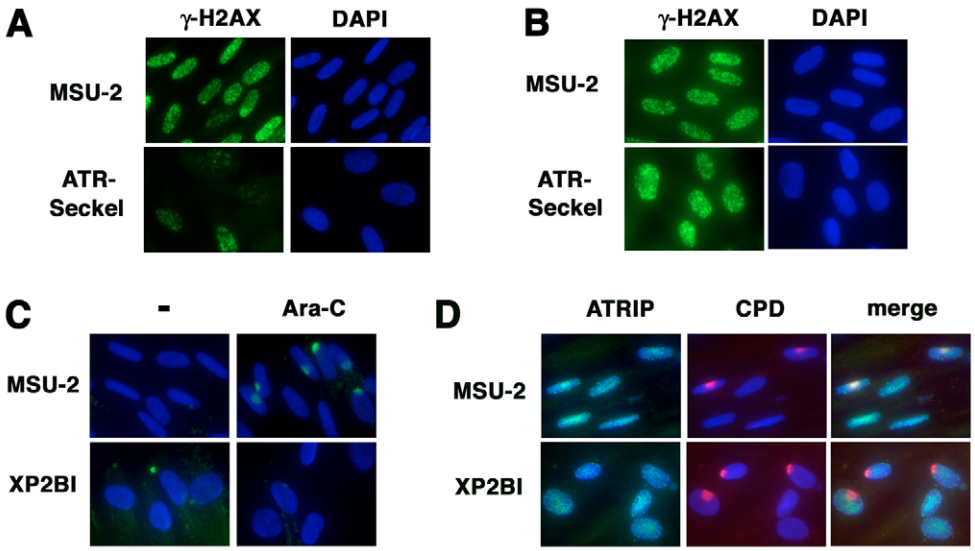

Fig. 6. ATR is the principal kinase for the NER-dependent H2AX phosphorylation. (A,B) MSU-2 or GM18366 (ATR-Seckel syndrome) cells under growth-arrested conditions were exposed to 10 J/m^2 UV and incubated for 4 hours (A), or treated with 40 μg/mL etoposide for 1 hour (B), and stained with anti-γ-H2AX antibody. (C,D) MSU-2 or XP2BI cells under growth-arrested conditions were exposed to local UV (40 J/m^2), and incubated for 1 hour in the presence or absence of 100 μM Ara-C before staining with anti-RPA2 (p34) antibody (C) or incubated for 1 hour with 100 μM Ara-C before double-staining with anti-ATRIP and anti-CPD antibodies (D).

Elledge, 2003). Taken together with the data that Ara-C treatment enhanced the accumulation of RPA and ATRIP at UV-damaged sites (Fig. 6C,D), we suggest that ssDNA-gap intermediates or their processed products are coated by RPA, which in turn recruits ATR-ATRIP to those sites (Fig. 8).

In budding yeast, a disruption of *RAD14*, the homolog of human *XPA*, exhibited a specific defect in activating checkpoint responses (phosphorylation of Rad53, Rad9 and Ddc2) following UV exposure, but not in the responses to methyl methane sulfonate (MMS) and DSB, suggesting physical and functional interactions between NER and the DNA damage checkpoint (Giannattasio et al., 2004). In humans, however, UV-induced checkpoint responses are normally or more effectively activated in NER-deficient XP-A cells, based on the phosphorylation and accumulation of p53 (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996) as well as the recruitment of RPA, Rad9 and ATR to damaged sites (Jiang and Sancar, 2006). The role of NER in damage checkpoint activation in human cells is still controversial. Recently, it has been reported that ATR signaling induced by UV or 4-nitroquinoline oxide, but not aphidicolin or IR, is compromised in XPA-deficient cells during S phase, as shown by defects in UV-induced ATRIP translocation to damaged sites, Chk1 and RPA phosphorylation, and RPA binding to chromatin (Bomgarden at al., 2006). The authors suggest that XPA plays a crucial role in the ATR-mediated checkpoint activation, independent of its function in NER. However, the UV-induced H2AX phosphorylation in quiescent cells observed here is absolutely dependent on NER (Fig. 3) and is not accompanied by Chk1 phosphorylation (Fig. 7A). We speculate that the NER-dependent ATR activation might be a local event at damaged DNA sites and does not go beyond H2AX to at least Chk1. However, it has been shown by immunostaining that p-Chk1(S317) is detectable in non-cycling primary human fibroblasts following UV in a dose-dependent manner (Stiff et al., 2005). The phosphorylation of Chk1 in quiescent cells, if any, should be very restricted since the cellular level of Chk1 in those cells is much lower than that in actively growing cells (Fig. 7A) or almost undetectable (Chini and Chen, 2003). Further analyses on other ATR substrates and identification of the factors colocalizing with γ-H2AX would be helpful for understanding the downstream reaction of the phosphorylation in quiescent cells.

Here we found that 53BP1 accumulates at locally UV-

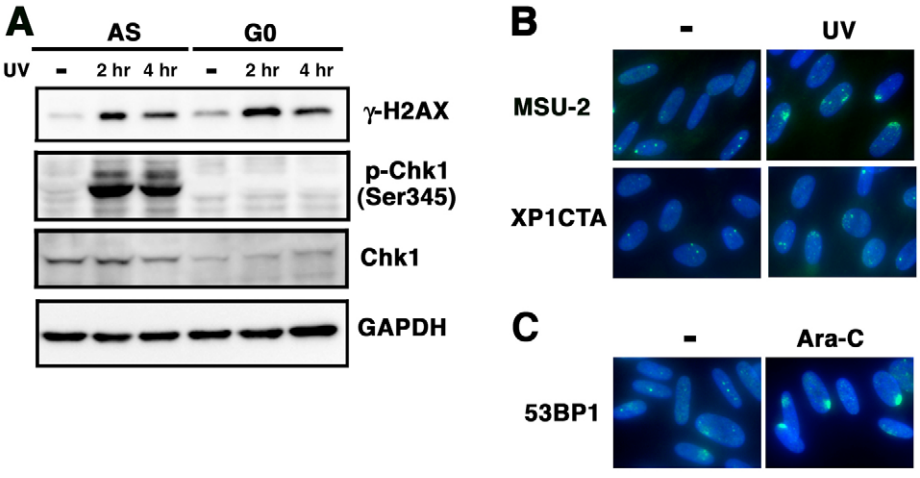

Fig. 7. The NER-dependent H2AX phosphorylation coincides with 53BP1 accumulation at damaged sites but not with Chk1 Ser345 phosphorylation. (A) MSU-2 cells (AS or G0) were exposed to 20 J/m^2 UV and incubated for 2 or 4 hours. Cell lysates were prepared with SDS sample buffer (Bio-Rad) and analyzed by immunoblotting with the specific antibodies as indicated. (B) MSU-2 or XP1CTA cells under growth-arrested conditions were locally irradiated with 20 J/m^2 UV and stained with anti-53BP1 antibody after 4 hours of incubation. (C) Growth-arrested MSU-2 cells were locally irradiated with 10 J/m^2 UV, incubated for 1 hour in the presence or absence of Ara-C and stained with anti-53BP1 antibody.

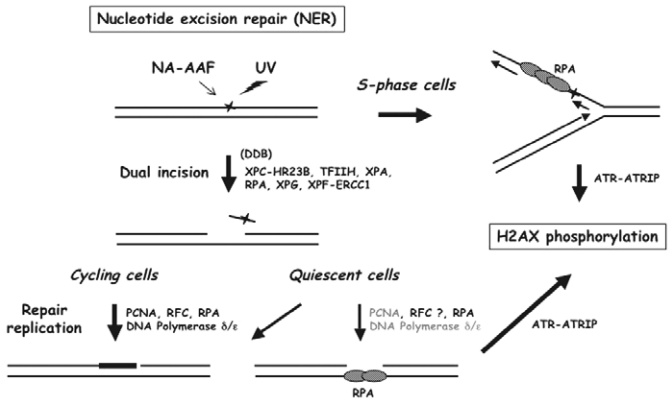

Fig. 8. Working model for the NER-dependent H2AX phosphorylation in G0/G1-arrested human cells. Two distinct but partially overlapping pathways can be activated by UV or NA-AAF: one is a replication stress-induced pathway in cycling S-phase cells (Ward and Chen, 2001; Ward et al., 2004) and the other is a NER-mediated pathway in quiescent cells (this study). In both pathways ATR is the principal kinase for the H2AX phosphorylation and RPA seems to play a role in the recruitment of ATR-ATRIP. A major difference between the two pathways is in how RPA-coated ssDNA regions are generated. The NER-mediated ssDNA gaps or their processed products might be caused by perturbed repair synthesis because of extremely low levels of replication factors including Pol δ, Pol ϵ and PCNA (shown in grey).

damaged subnuclear regions of quiescent cells (Fig. 7B,C). IR-induced γ-H2AX foci are known to colocalize with various factors involved in DSB repair or damage signaling, including BRCA1, Nbs1, Rad50, Rad51, 53BP1 and MDC1 (Paull et al., 2000; Schultz et al., 2000; Rappold et al., 2001; Goldberg et al., 2003; Lou et al., 2003; Stewart et al., 2003). HU treatment induces nuclear foci of 53BP1 and BRCA1 colocalizing with γ-H2AX foci in S-phase cells (Ward and Chen, 2001). So far we have not succeeded in detecting the local accumulation of other factors including BRCA1 following physiological doses of UV (M.M. and T.M., unpublished). Cleaver and his colleagues have also recently reported panstaining (but not foci) of 53BP1 in G1-phase cells after global UV irradiation (Marti et al., 2006). The role of 53BP1 in the signaling pathway is currently unknown and awaits further studies.

Our findings provide a new insight into the quality control of the DNA repair process. The phosphorylation of histone H2AX might be implicated in the cellular response to the interrupted NER caused by the reduced gap-filling activity. Like NER, other repair systems containing a step of DNA synthesis by Pol δ and/or Pol ϵ also have a chance to generate ssDNA-gap intermediates in quiescent cells. In addition to UV and NA-AAF, MMS and hydrogen peroxide also seem to induce H2AX phosphorylation in quiescent human primary fibroblasts (Jessica A. Downs, Department of Biochemistry, University of Cambridge, UK; personal communication). The ssDNA gaps in the genome can be enzymatic targets for further processing (e.g. endonucleolytic or exonucleolytic cleavage) and potentially induce genomic instability, unless the gaps are protected until repair replication takes place or are filled by other mechanisms. We emphasize that this situation would be more considerable for in vivo cells since most of those are in a non-cycling state. In fact, model experiments using peripheral T lymphocytes that are typical non-cycling G0 populations reproducibly exhibited a significant level of H2AX phosphorylation following UV (see supplementary material Fig. S5). Whether those cells in mice treated with chemicals producing DNA lesions in vivo exhibit the phosphorylation or not is under investigation.

Materials and Methods

Cell culture and UV irradiation

Human primary fibroblasts MSU-2 (normal), TIG-120 (normal), XP12BE (XP complementation group A), XP1CTA (XP complementation group C), XP2BI (XP complementation group G), GM18366 (ATR-Seckel syndrome) and AT2KY (ataxia-telangiectasia) were used in this study and grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% FBS (Sigma) and 50 μg/mL gentamicin at 37°C in a humidified incubator under 5% CO_2. MSU-2 and XP12BE were kindly provided by James E. Trosko (Michigan State University) and XP1CTA was a generous gift from Mituo Ikenaga (Kyoto University) (Wang et al., 1991). XP2BI (GM03021) and GM18366 were obtained from Coriell Cell Repositories (Camden, NJ), and TIG-120 and AT2KY were from the Human Science Research Resources Bank (Osaka, Japan). An immortal XP-A cell line XP3OS/T-n was obtained by introducing the hTERT gene into XP3OS cells (Takebe et al., 1977) as described previously (Nakamura et al., 2002). To arrest the cells in G0 phase, cells were cultured for 4 days on glass-bottom culture dishes or 100-mm culture dishes, refed with medium containing 0.5% FBS and maintained for a further 3 or 4 days.

Cells grown on culture dishes were washed twice with PBS(–) and irradiated with 254-nm UV from germicidal lamps (GL-10, Toshiba). For micropore UV irradiation, cells were covered with an isopore polycarbonate membrane filter (pore size 8 μm, Millipore) and exposed to UV (Katsumi et al., 2001). In some experiments, cells were treated with Ara-C (Sigma) during pre- and/or post-UV incubation as described previously (Stiff et al., 2005). Etoposide and NA-AAF were purchased from Sigma and Midwest Research Institutes (Kansas City, MO), respectively.

Antibodies and immunostaining

Specific antibodies employed in this study were rabbit polyclonal anti-γ-H2AX (Trevigen) and anti-ATRIP (Affinity BioReagents), rabbit monoclonal anti-phospho-Chk1 (Ser345) (Cell Signaling), and mouse monoclonal anti-γ-H2AX (Upstate), anti-Pol δ 125-kDa CS and anti-Pol ϵ 261-kDa CS (BD Biosciences), anti-53BP1 (Iwabuchi et al., 2003), anti-Chk1 (Santa Cruz) and anti-RPA p34 subunit (Ab-2) and anti-PCNA (Ab-1) (Oncogene Research). Monoclonal antibody specific for human XPA (A-2) was obtained by immunizing mice with the fusion proteins of XPA and glutathione S-transferase.

Cells grown on 35-mm glass-bottom culture dishes were fixed either in 4% formaldehyde for 15 minutes or in methanol/acetone (1:1) at –20°C for 10 minutes. In the case of formaldehyde fixation, cells were subsequently treated with ice-cold 0.2% Triton X-100 in 10 mM PBS (pH 7.4) on ice for 10 minutes and acetone at –20°C for 5 minutes. After blocking with 10 mM PBS (pH 7.4) containing 20% FBS at 37°C for 30 minutes, cells were incubated with an appropriate primary antibody and then with secondary antibody [Alexa Fluor-488 goat anti-mouse immunoglobulin G (IgG) (H+L) conjugate or anti-rabbit IgG (H+L) conjugate (Molecular Probes)], and counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes) for 5 minutes. For RPA or ATRIP staining, cells were treated with ice-cold 0.5% Triton X-100 in 10 mM PBS (pH 7.4) on ice for 2 or 5 minutes before fixation, respectively. A Leica DMIRBE microscope equipped with a cooled CCD camera (CoolSNAP HQ, Photometrics) was used to obtain fluorescence images.

To test the colocalization of γ-H2AX and UV-induced DNA lesions, UV-irradiated cells were stained with anti-γ-H2AX and Alexa Fluor-488 goat anti-rabbit IgG (H+L) conjugate as described above and refixed in 2% formaldehyde for 5 minutes. After treatment with 2 M HCl for 5 minutes to denature DNA, cells were incubated with TDM-2 antibody specific for CPD (Mori et al., 1991) and subsequently with Alexa Fluor-594 goat anti-mouse IgG (H+L) conjugate.

In order to distinguish S-phase cells from others, cultured cells were labeled with BrdU for 15 minutes before fixation. After staining with appropriate primary and secondary antibodies as described above, cells were refixed in 2% formaldehyde, treated with 2 M HCl at 37°C for 20 minutes and incubated with Alexa Fluor-594 anti-BrdU conjugate (Molecular Probes).

Flow cytometry

Cells were fixed in methanol/acetone (1:1) at –20°C for 10 minutes and incubated with FACS wash solution [0.1% bovine serum albumin, 2 mM EDTA in PBS(–)] containing 50 μg/mL RNase A (Sigma) at 37°C for 30 minutes. Cells were stained with γ-H2AX antibody followed by Alexa Fluor-488 goat anti-mouse IgG (H+L) conjugate. In the case of BrdU-labeling experiments, cells were further processed as described above and incubated with Alexa Fluor-647 anti-BrdU conjugate (Molecular Probes). After counterstaining with 25 μg/mL PI (Sigma), cells were analyzed using FACS-Calibur (Becton Dickinson).

Western blotting

Cells were lysed in NP-40 lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, protease inhibitor cocktail (Roche), 1% phosphatase inhibitor cocktails 1 and 2 (Sigma)] on ice for 30 minutes and centrifuged at 16,100 ***g*** at 4°C for 15 minutes. Aliquots of the supernatants were boiled in SDS sample buffer (Bio-Rad), separated on SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). After blocking with PBST [10 mM PBS (pH 7.4), 0.05% Tween 20] containing 5% non-fat dry milk, the membrane was incubated with primary antibody followed by secondary antibody conjugated with horseradish peroxidase or alkaline phosphatase, and the signals were detected with Super Signal West Femto reagent (Pierce) or BCIP/NBT color substrate (Promega), respectively.

In vivo repair assay

Cells were irradiated with 10 J/m^2 UV and cultured for various periods in a humidified 37°C incubator under 5% CO_2. Genomic DNA was isolated using DNeasy kit (Qiagen) and the amounts of CPD or 6-4PP were determined by an enzyme-linked immunosorbent assay using the specific monoclonal antibodies TDM-2 or 64M-5, respectively (Mori et al., 1991).

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