

# Replication-dependent and -independent Responses of RAD18 to DNA Damage in Human Cells\*

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Postreplication repair facilitates tolerance of DNA damage during replication, overcoming termination of replication at sites of DNA damage. A major post-replication repair pathway in mammalian cells is translesion synthesis, which is carried out by specialized polymerase(s), such as polymerase  $\eta$ , and is identified by focus formation by the polymerase after irradiation with UVC light. The formation of these foci depends on RAD18, which ubiquitinates PCNA for the exchange of polymerases. To understand the initial processes in translesion synthesis, we have here analyzed the response to damage of RAD18 in human cells. We find that human RAD18 accumulates very rapidly and remains for a long period of time at sites of different types of DNA damage, including UVC light-induced lesions, and x-ray microbeam- and laser-induced single-strand breaks, in a cell cycle-independent manner. The accumulation of RAD18 at DNA damage is observed even when DNA replication is inhibited, and a small region containing a zinc finger motif located in the middle of RAD18 is essential and sufficient for the replication-independent damage accumulation. The zinc finger motif of RAD18 is not necessary for UV-induced polymerase  $\eta$  focus formation, but another SAP (SAF-A/B, Acinus and PIAS) motif near the zinc finger is required. These data indicate that RAD18 responds to DNA damage in two distinct ways, one replication-dependent and one replication-independent, involving the SAP and zinc finger motifs, respectively.

DNA in living cells is highly vulnerable to endogenous and environmental-damaging agents. If not accurately repaired, DNA damage may cause mutation and cell death, and can eventually lead to cancer and aging (1). In human cells the majority of DNA damage appears to be repaired by excision repair pathways (2). However, considerable amounts of damage are thought to escape repair because of the limited repair capacity of cells. During DNA replication, unrepaired DNA damage may inhibit the progression of replication forks, and this inhibition may result in gaps in the newly synthesized strand and may be

converted to more severe secondary damage, such as DNA double-strand breaks (DSB)<sup>2</sup> (3). Recently, it has become clear that cells, from bacteria to human, possess highly significant postreplication repair mechanisms (PRR) to overcome such replication blockages (4, 5).

In the yeast *Saccharomyces cerevisiae*, a number of genes belonging to the RAD6 epistasis group are responsible for the PRR pathway (6). RAD6 and RAD18 are two of the most important genes involved in the PRR pathway and mutation of either gene results in hypersensitivity to UV light and loss of UV-induced mutagenesis, indicating that Rad6 and Rad18 are involved in error-free as well as error-prone modes of PRR (7). Rad6 is a ubiquitin-conjugating enzyme (E2), and the functions of Rad6 in yeast depend on its ability to transfer ubiquitin molecules to substrate proteins (8–10). Although Rad6 interacts with several ubiquitin-ligating enzymes (E3), the interaction with Rad18 is essential for carrying out PRR (10–13). The N-terminal region of Rad18 contains a RING finger motif, which confers ubiquitin ligase activity (14). The middle part of Rad18 contains a C2HC type zinc finger motif and a SAP domain, which are thought to mediate protein-protein interaction or DNA binding (15, 16). The C-terminal region of Rad18 is required for its interaction with Rad6 (17). The yeast Rad18 has single-stranded DNA (ssDNA) binding activity, and the majority of cellular Rad18 forms a highly stable binary complex with Rad6 (11, 18). Because Rad6 does not have any DNA binding activity and interacts tightly with Rad18, it has been proposed that Rad18 recruits Rad6 to the site of DNA damage via its physical interaction, where Rad6 modulates the function of stalled DNA replication machinery with its ubiquitin-conjugating activity (11). Supporting this hypothesis, it has been reported that proliferating cell nuclear antigen (PCNA), a DNA polymerase sliding clamp involved in DNA synthesis and repair, is ubiquitinated in a Rad6- and Rad18-dependent manner and the monoubiquitination of PCNA is necessary for carrying out translesion synthesis by polymerase  $\eta$  (Pol $\eta$ ) in yeast cells (19–21). However, how Rad18 is targeted to the stalled replication forks remains to be understood.

The genes responsible for PRR are highly conserved throughout evolution. In mammals, the Rad6 homolog is encoded by

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<sup>2</sup> The abbreviations used are: DSB, double-strand break; PCNA, proliferating cell nuclear antigen; nt, nucleotide; GFP, green fluorescent protein; PBS, phosphate-buffered saline; WT, wild type; ssDNA, single-strand DNA; Gy, Gray; PRR, postreplication repair mechanisms; SSB, single-stranded break; NER, nucleotide excision repair; DAPI, 4',6'-diamidino-2-phenylindole.

## Accumulation of RAD18 at Sites of DNA Damage

two closely related genes, *HHR6A* and *HHR6B*, each of which complement the defects in DNA repair and UVC light mutagenesis of the yeast *rad6* mutant (22). Neither mHR6A nor mHR6B knock-out mouse embryonic fibroblasts show increased UVC light sensitivity in contrast to the severe UVC light sensitivity of the Rad6-deficient yeast cells, indicating a functional redundancy between the two proteins in DNA repair (23, 24). On the other hand, only one gene, whose gene product shows amino acid sequence similarity to yeast Rad18, is present in mammals (25, 26). Human RAD18 protein can interact with both HHR6A and HHR6B, and either overexpression of a dominant negative RAD18 mutant protein in human cells or targeted disruption of the *Rad18* gene in mouse embryonic stem cells results in increased UVC light sensitivity, suggesting that mammalian RAD18 is involved in PRR (25, 27). Recently, it was shown that human PCNA is also monoubiquitinated in a RAD18-dependent manner at stalled replication forks by UV light lesions, and this might cause a polymerase switch from replicative polymerase to the translesion polymerase, Pol $\eta$  (28, 29). These data suggest that the mode of PRR is conserved from yeast to mammals.

To understand the step required before monoubiquitination, in other words, how human RAD18 is recruited to the site of damage, we analyzed damage-dependent translocation of RAD18 in human cells. Here, we show that human RAD18 accumulates at the site of UVC light-induced DNA damage and UVA laser-induced DNA single-strand breaks (SSB) independently of DNA replication and dependent on the zinc finger motif of RAD18. This zinc finger motif is dispensable for Pol $\eta$  focus formation. However, we found that deletion of the SAP domain located near the zinc finger motif of RAD18 abrogates its complementation ability for UV-induced Pol $\eta$  focus formation in RAD18-deficient cells, suggesting that RAD18 may assemble at the site of stalled replication forks via its SAP motif.

### EXPERIMENTAL PROCEDURES

**Cell Lines and Culture Conditions**—HeLa cells were used unless otherwise indicated. The nucleotide excision repair (NER)-deficient cell line was XP12ROSV, an SV40-transformed XP-A cell line derived from an XP-A patient. The RAD18-knock-out cell line established from human HCT116 cell lines will be reported elsewhere.<sup>3</sup> Cells were cultured in Eagle's minimal essential medium containing 10% fetal calf serum. Synchronization of cells was performed by the double thymidine block method. Briefly,  $5 \times 10^4$  cells were seeded into 3.5-cm dishes and grown for 2 days. Thymidine was then added to 2.5 mM final concentration, and cells were further incubated for 22–24 h. Thymidine-containing medium was removed and cells were washed three times with Hank's buffer and fresh medium was added. After 10 h of incubation, hydroxyurea (HU) was added to 1 mM final concentration, and cells were incubated for 14–16 h. Under these conditions, cells accumulate at the G<sub>1</sub>/S border. HU-containing medium was removed, and cells were washed three times with Hank's buffer, and the cells were incubated for 0, 3, 8, and 16 h in fresh medium to obtain G<sub>1</sub>/S, S-phase, G<sub>2</sub>-M, G<sub>1</sub>-phase cells, respectively. Synchroni-

zation of the cell cycle was confirmed by FACSCalibur (Becton Dickinson) and immunostaining for the incorporation of BrdU.

**Plasmids**—The human RAD18 open reading frame was amplified by PCR from a HeLa cell cDNA library using PCR primers with a XhoI site at the 5' terminus and a NotI site at the 3' terminus and cloned into pT7Blue (Novagen). The sequence of the cloned gene was confirmed by sequencing. GFP-tagged RAD18 was generated by an in-frame ligation of a RAD18 fragment (4–1485 nt) encoding the entire RAD18 sequence, except for the start and termination codons, into either pEGFP-C1 or N1 or pDsRed-Monomer-C1 (Clontech). To generate FLAG-tagged RAD18 protein, the same fragment was introduced into a pCY4B-N-FLAG mammalian expression vector harboring a chicken  $\beta$ -globin promoter and an N terminus FLAG tag. To generate GFP-tagged RAD18 deletion proteins, pEGFP-C1-RAD18 was digested with restriction enzymes and appropriately ligated to produce in-frame fusions. To delete the N terminus of the RAD18 protein, a RAD18 fragment (580–1485 nt) was amplified by PCR and cloned into pEGFP-C1. To generate pEGFP-RAD18 1–449CF, pEGFP-RAD18 was amplified by PCR with the following primers: 5'-TGTCCTGTTTGGGG-GTTAACATT-3' and 5'-ATCCACTTTAGTAACTTGTTTCAA-3'. To generate pEGFP-RAD18  $\Delta$ 248–284, the 5' fragment of RAD18 cDNA was obtained by PCR with the following primers: 5'-CTCGAGGACTCCCTGGCCGAGTCTCGGTG-GCCT-3' and 5'-GAATTCTACAGTTTTGGGCAGCGGCT-TCCCTTTT-3' and the 3' fragment of RAD18 cDNA was obtained by PCR with the following primers: 5'-GAATTCCA-CATGTACAATGCCCAATGCGATGCT-3' and 5'-GCGGC-CGCAATTCTATTACGCTTGTTC-3'. These fragments were cloned into pT7Blue and digested with XhoI and EcoRI or EcoRI and NotI, respectively. These fragments were introduced into pBluescript II and digested with XhoI and NotI, then inserted into the XhoI - NotI sites of pEGFP-C1. All the constructs were confirmed by sequencing.

**Local UVC Light Irradiation**—Local UVC light irradiation was performed as previously described (30). Briefly, cells were plated at  $1 \times 10^5$  cells per 3.5-cm glass bottom dish (poly-L-lysine-coated; MATSUNAMI) and cultured for 2 days. Cells were then washed twice with Hank's buffer and covered with a polycarbonate isopore membrane filter with pores of 3  $\mu$ m in diameter (Millipore) and irradiated with 10–100 J/m<sup>2</sup> UVC light. After irradiation, cells were incubated in growth medium for the time indicated.

**Micro X-ray Beam Irradiation**—We used a synchrotron radiation light source installed in the Photon Factory, Institute of Materials Structure Science, High Energy Accelerator Research Organization (KEK) in Tsukuba, Japan. Cells were irradiated with an x-ray microbeam, which was focused with a metal slit system onto a 3–5  $\mu$ m square. The intensity of X-rays obtained with the slit system is about 40 R/s. Experimental details will be published elsewhere (31).

**Antibodies**—The rabbit antibody to human RAD18 has been described previously (25). Mouse monoclonal antibody (Anti-FLAG M2; Sigma) was used for observation of FLAG-tagged protein. A mouse monoclonal antibody (Anti-BrdU; Roche) was used for detection of DNA synthesis. The antibody for detection of UVC light-induced DNA damage was TDM2,

<sup>3</sup> M. Mori and T. Shiomi, manuscript in preparation.

which is specific for cyclobutane pyrimidine dimers (CPD) (32). XRCC1 Ab-1 (33–2-5) (Neo Markers) and PCNA (Ab-1) (Oncogene) were used. Secondary antibodies were Alexa Fluor 488 goat anti-mouse, Alexa Fluor 594 goat anti-mouse, and Alexa Fluor 594 goat anti-rabbit (Molecular Probes).

**Immunofluorescence**—Cells were washed twice with PBS, then fixed with 4% paraformaldehyde for 1 h. Cells were washed once for 5 min with TNT buffer (0.1 M Tris-HCl, pH 7.5, 1.5 M NaCl, 0.05% Tween 20) and permeabilized in 0.2% Triton X-100/PBS for 5 min and washed three times for 5 min with TNT buffer. Cells were incubated in TNB buffer (TNT buffer containing blocking reagent, PerkinElmer Life Sciences) for 30 min to block nonspecific binding. Primary and secondary antibodies were diluted in TNB buffer and incubated for 1 h. After each antibody binding step, cells were washed three times for 5 min with TNT buffer. DNA was stained with 4',6'-diamino-2-phenylindole (DAPI). When staining for CPD, DNA was denatured by incubation in 2.5 N HCl for 5 min at 37 °C after Triton X-100 treatment and cells were washed twice for 5 min with PBS and then incubated in TNB buffer. For observation of GFP-tagged RAD18 and CPD, cells were washed twice with PBS, then fixed with methanol-acetone for 10 min at –20 °C. Cells were washed for 5 min with TNT buffer and incubated in TNB buffer after fixation without HCl treatment. To monitor DNA synthesis, cells were incubated in 10  $\mu$ M BrdU for 30 min before fixation. After fixation, cells were treated with 2.5 N HCl at 37 °C for 1 h, then washed three times for 5 min with PBS, once for 5 min with TNT buffer and incubated in TNB buffer. Incubation was performed at room temperature except for denaturing of DNA.

**Microscopy and Laser Microirradiation**—Cells were transfected with plasmid DNA by FuGene6 (Roche Applied Science) and cultured for 2 days. Before UVA laser irradiation, the growth medium was changed to OPTI-MEM (Invitrogen), and cells were placed into a humidity chamber on the microscope stage (OLYMPUS) to keep the temperature at 37 °C. Confocal fluorescence images were captured using a FV500 laser-scanning microscope (OLYMPUS). We used UVA laser irradiation either passed through a F20 filter, which penetrates 0.75  $\mu$ J light per single pulse and produces mainly SSB for observation of accumulation of GFP-RAD18 or through a F25 filter, which penetrates 2.5  $\mu$ J light per single pulse and produces SSB as well as DSB for observation of accumulation of endogenous RAD18. Further details of UVA laser microirradiation have been described previously (33).

## RESULTS

**Accumulation of Human RAD18 at UVC Light-irradiated Sites**—To investigate the response of RAD18 to DNA damage, we exposed cells to local irradiation using two different sources of light, local UVC light and a UVA laser, which produce cyclobutane pyrimidine dimers (CPD) and 6–4 photoproducts (6–4PP), or SSB, DSB, and oxidative base lesions, respectively (30, 33–35). We first examined the response of RAD18 to UVC light in HeLa cells by local UVC light irradiation. Cells on a dish were covered with a polycarbonate filter with micropores of 3- $\mu$ m diameter prior to UVC light irradiation. Because the filter blocks UVC light with wavelengths shorter than 300 nm, UVC

light can pass only through the pores and produce DNA lesions at several discrete spots in the nuclei of the irradiated cells. As shown in Fig. 1A, CPD, one of the major UVC light-induced DNA lesions, was detected in localized spots after UVC light irradiation of 100 J/m<sup>2</sup> through filter pores. Because CPD are repaired only slowly by NER even in wild-type human cells (36), the intensity of the spots for CPD decreased only slightly even 8 h after irradiation (*upper panel* in Fig. 1A). Before UVC light irradiation, endogenous RAD18 molecules were detected by RAD18 antibody in the nucleus as small dots (*No UVC* in Fig. 1A). After 15 min of postirradiation incubation, RAD18 had already accumulated at the irradiated sites, and co-localized with the CPD spots as shown in the merged images in the panel. RAD18 was observed co-localized with CPD until 8 h after local UVC light irradiation with 100 J/m<sup>2</sup>. After local UVC light irradiation with the much lower dose of 10 J/m<sup>2</sup>, spots of RAD18 were detected as well (Fig. 1B). To determine the effect of NER, we investigated the accumulation of RAD18 in NER-deficient XP-A cells. As shown in Fig. 1C, RAD18 accumulated at the sites of DNA damage and co-localized with CPD in the absence of XPA. Because similar results were obtained in XP-C mutant cells (not shown), we concluded that RAD18 accumulates at UVC-irradiated regions independently of NER.

**Human RAD18 Accumulates at SSB Sites Produced by UVA Laser and X-ray Microbeams**—Because RAD18-deficient embryonic stem cells are sensitive to various DNA-damaging agents besides UVC light irradiation (27), RAD18 protein may respond to DNA damage other than UVC light-induced lesions. We recently established an experimental system to characterize real time responses of repair proteins to various types of oxidative DNA damage (33). This experimental system consists of a confocal laser scanning microscope and 365 nm UVA laser light irradiation equipment and can create several types of DNA damage in a restricted area of the nucleus in a laser dose-dependent manner. We have shown that after irradiation of cells with laser light passed through a F20 filter DNA lesions other than SSB were hardly detected. The laser light dose used in this study does not produce detectable UVC light-induced lesions; neither CPD nor 6–4PP, were detected by antibodies against the lesions and neither of the photolyases for CPD or 6–4PP accumulates at the irradiated sites (not shown). Fig. 2A shows that endogenous RAD18 protein is detected at irradiated sites and colocalized with XRCC1 (*upper panel*) and PCNA (*lower panel*), respectively, 30 min after irradiation with 365-nm UVA laser light of passed throughout a F25 filter. Thus, RAD18 accumulates at sites of laser-induced DNA damage.

We transiently expressed GFP-tagged RAD18 in HeLa cells and examined the kinetics of the accumulation of RAD18 at sites of damage. GFP-tagged RAD18 accumulated at irradiated sites after irradiation with a low laser dose, at which GFP-tagged XRCC1 but not other proteins such as DNA glycosylases for base damage or NBS1 for DSB accumulate (33), indicating that RAD18 accumulates at SSB induced by UVA laser light (Fig. 2B). Accumulation of RAD18 was detected around 2 min after irradiation and the intensity of GFP fluorescence at the irradiated site increased gradually, reaching plateau at around 10 min (Fig. 2C) and not decreasing significantly until at least 1 h after irradiation (not shown). We have previously shown



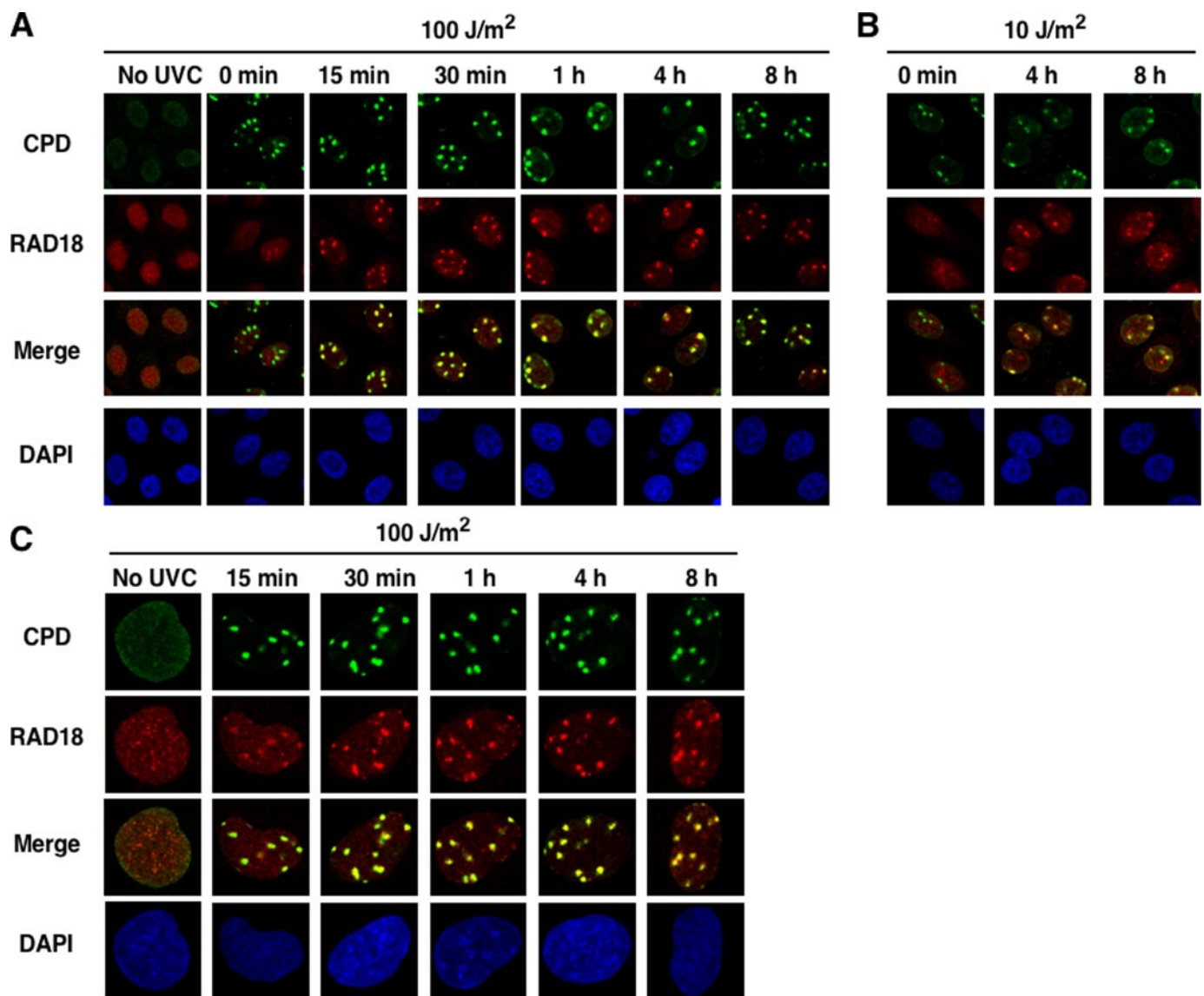


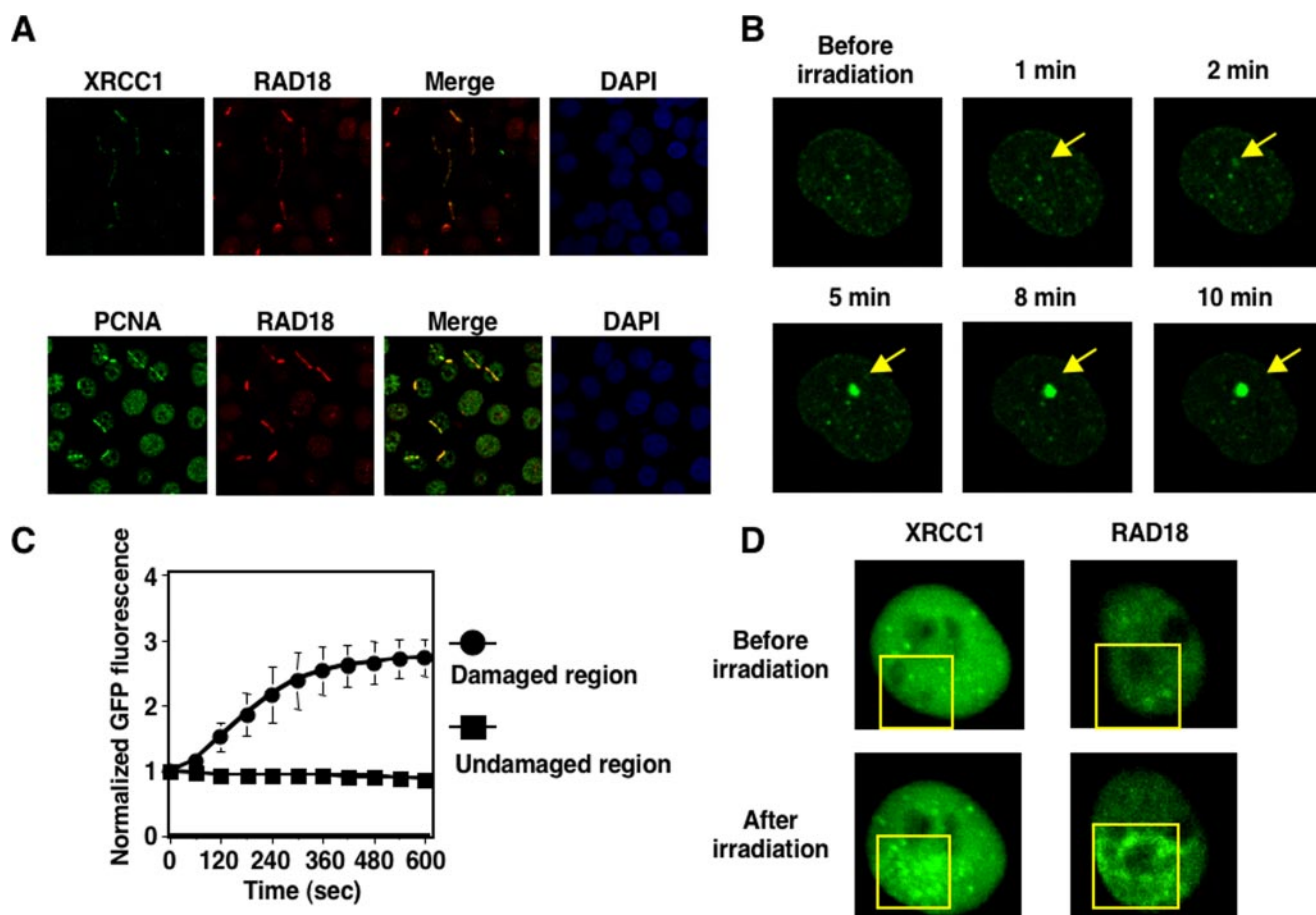
FIGURE 1. **RAD18 accumulates at sites of DNA damage induced by local UVC light.** A, HeLa cells were unirradiated or irradiated at 100 J/m<sup>2</sup> UVC light with a porous filter. Cells were fixed at the times indicated and stained with anti-RAD18 and anti-CPD antibodies and counterstained with DAPI. 0 min indicates the time immediately after irradiation. B, HeLa cells were irradiated at 10 J/m<sup>2</sup> UVC light with a porous filter. C, XP-A cells were irradiated at 100 J/m<sup>2</sup> UVC light with a porous filter.

that accumulation of XRCC1 is dependent on the activation of poly(ADP-ribose) polymerase at SSB and the accumulation of XRCC1 is significantly affected by prior treatment of cells with 1,5-dihydroxyisoquinoline (DIQ), a potent inhibitor of PARP activation (30, 33). In contrast to XRCC1, DIQ treatment did not affect the accumulation of GFP-RAD18 at all and accumulation of RAD18 was not influenced by the absence of XRCC1 in the Chinese hamster XRCC1 mutant cell line, EM9 (not shown), indicating that accumulation of RAD18 at SSB is not influenced by XRCC1-dependent SSB repair pathways.

To investigate whether RAD18 accumulates at SSB induced by agents other than UVA laser light, we irradiated cells with a microbeam of x-rays over a restricted area of the cell nuclei (31). As with UVA laser irradiation, GFP-tagged RAD18 accumulated at irradiated areas after irradiation with a dose at which we detected the accumulation of GFP-tagged XRCC1 but not RAD52 (Fig. 2D and not shown). The data correlate with the

results obtained by UVA laser irradiation and confirm that RAD18 accumulates at SSB produced by x-rays.

**Accumulation of Human RAD18 at Sites of DNA Damage Is Independent of DNA Replication**—Because RAD18 plays a pivotal role in PRR, it is believed that the function of RAD18 is associated with DNA replication (11). We, therefore, asked whether the accumulation of human RAD18 at the site of DNA damage depends on DNA replication. Cells were synchronized at the G<sub>1</sub>/S border by the double thymidine block method and released by removing HU-containing medium. Cells from different cell cycle phases were irradiated with UVC light through a porous filter or with a UVA laser. Although accumulation of RAD18 is most efficient in S-phase cells, RAD18 also accumulated in G<sub>1</sub>/S-arrested and G<sub>1</sub>-phase cells and remained at UVC light-irradiated sites for a long period (Fig. 3A and not shown). Whereas the frequency of RAD18 foci, which co-localized with CPD, decreased slightly in G<sub>2</sub>-M phase, accumulation of



**FIGURE 2. RAD18 accumulates at sites of DNA damage induced by UVA laser irradiation.** *A*, HeLa cells were irradiated with UVA laser through a lens. At 30 min postirradiation, cells were fixed and stained with anti-RAD18 and anti-XRCC1 or anti-RAD18 and anti-PCNA antibodies, respectively, and counterstained with DAPI. *B*, accumulation of GFP-tagged RAD18 after irradiation with UVA laser in HeLa cells at indicated time points is shown. *C*, kinetics of GFP-tagged RAD18 accumulation at laser-induced DNA damage. The results show the average from three independent experiments, and the error bars indicate S.D. *D*, accumulation of GFP-tagged XRCC1 and RAD18 after irradiation with an x-ray microbeam in HeLa cells. The square indicates the irradiated site. HeLa cells expressing GFP-tagged XRCC1 or RAD18 were irradiated with an x-ray microbeam at 20 Gy or 80 Gy, respectively, and accumulation observed at around 10 min after irradiation.

RAD18 was observed in all cell cycle phases. To confirm that the accumulation of RAD18 at the site of DNA damage does not depend on DNA replication, we visualized DNA replication by immunostaining for incorporated BrdU. In the presence of HU, DNA replication was inhibited and nuclei were not stained by the anti-BrdU antibody. After removal of HU, DNA replication initiated and more than 80% of cell nuclei were stained with anti-BrdU antibody at high levels (Fig. 3*B*). Although these nuclei were stained with anti-BrdU antibody at only background levels, accumulation of RAD18 was observed at the site of damage induced by local UVC irradiation (Fig. 3*C*), indicating that accumulation of RAD18 occurs even in cells not in S phase. As in the case of UVC light irradiation, GFP-tagged RAD18 also accumulated at sites of UVA laser irradiation in all cell cycle phases (not shown). The kinetics and intensity of accumulation of RAD18 were the same in cells at different phases of the cell cycle (not shown). These results indicate that the accumulation of RAD18 at the site of UVC light lesions and SSB is independent of DNA replication.

**Identification of the Minimal Domain of RAD18 Required for Accumulation**—To identify the region of RAD18 required for the accumulation at the sites of DNA damage, we transiently

expressed a series of RAD18 deletions fused with GFP at their N terminus in HeLa cells (Fig. 4*A*). The N-terminal region of RAD18 contains a RING finger motif, and the middle region contains a zinc finger motif and SAP domain. The C-terminal region has a basic and an acidic domain, which are necessary for the interactions with HHR6 and Pol $\eta$  (29). RAD18 has three putative nuclear localization signals (238–242, 343–347, 488–494) identified by PSORT. Full-length RAD18 localized exclusively in the nucleus, but the mutant (RAD18-(1–449)) lacking an NLS in the C terminus is found both in the nucleus and in the cytoplasm (Fig. 4). Further deletion of the C terminus containing the second putative NLS restored the nuclear localization (RAD18-(1–282) in Fig. 4*A*). All the mutant proteins, except those lacking the zinc finger motif (RAD18-(1–209), RAD18-( $\Delta$ 209–449)), accumulated at the sites of DNA damage induced by both UVA laser and UVC light (Fig. 4). These data indicate that the zinc finger motif is essential for its accumulation. To confirm whether the zinc finger motif is responsible for accumulation, we replaced the second conserved cysteine with phenylalanine within the zinc finger motif (RAD18-(1–495CF)). Like deletion mutants lacking the zinc finger motif, RAD18-(1–495CF) did not accumulate at the sites of DNA damage induced by both UVA laser and UVC light (Fig. 4).

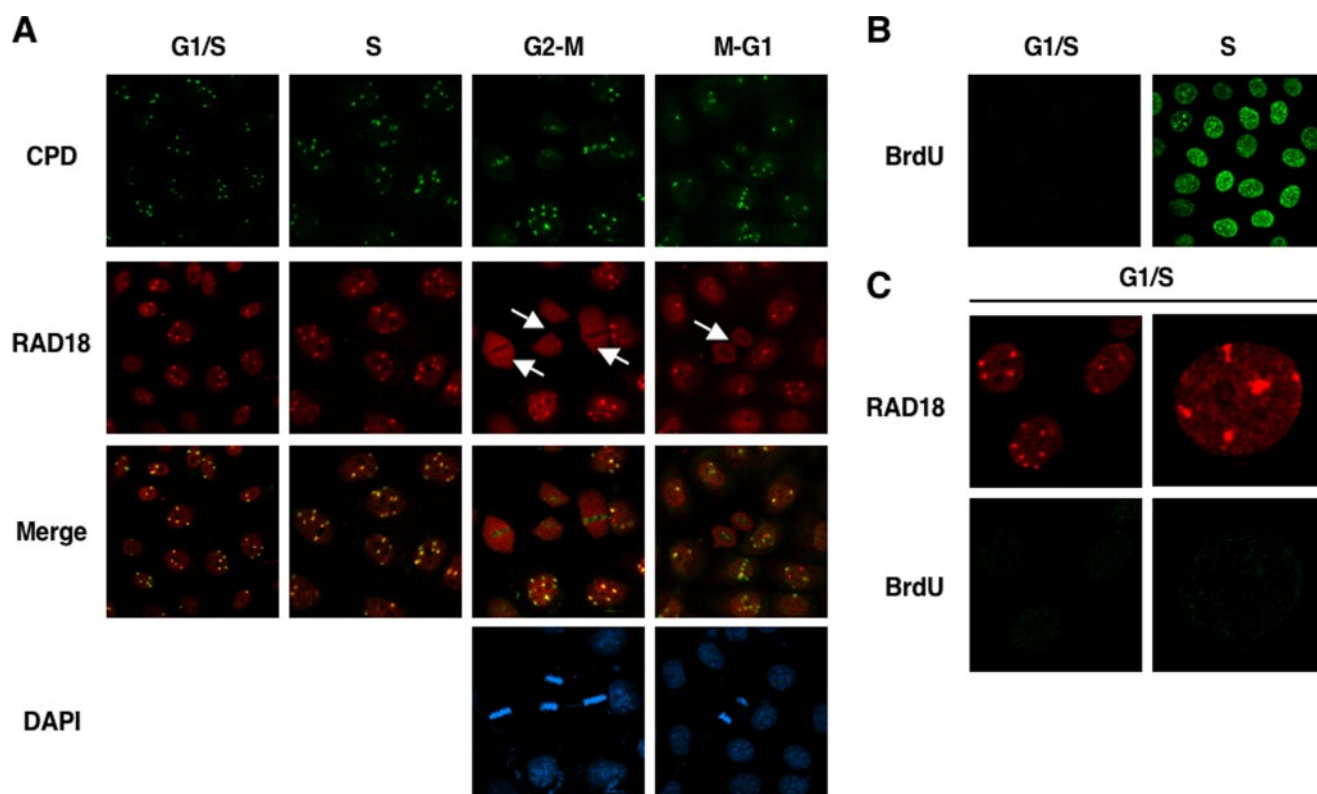


FIGURE 3. **RAD18 accumulates at sites of DNA damage independently of DNA replication.** A, HeLa cells were synchronized, and cells from each cell cycle phase were then irradiated at 100 J/m<sup>2</sup> UVC light through a porous filter. Cells were incubated for 1 h or the time indicated, prior to fixing and staining with anti-RAD18 and anti-CPD antibodies. The *arrow* indicates mitotic cells. B, immunostaining for incorporated BrdU. To visualize DNA replication, HeLa cells were labeled for 30 min with BrdU in the presence of HU or at 3 h after removal of HU, respectively. C, HeLa cells were synchronized at the G<sub>1</sub>/S border and irradiated with 100 J/m<sup>2</sup> UVC light through a porous filter. Cells were incubated for 1 h in the presence of HU, then fixed, and the accumulation of RAD18 examined.

These data suggest that a domain containing the zinc finger motif is important for the accumulation of RAD18 at irradiated sites. Because it has been reported that yeast or human RAD18 interacts with itself through the zinc finger domain (37, 38), we, therefore, introduced the middle region of RAD18 harboring 194–282 into RAD18-deficient human cell and found that RAD18-(194–282) accumulated at sites of UVA laser-induced damage (Fig. 4B). From these data we concluded that a small region containing the zinc finger motif is essential and sufficient for the accumulation of RAD18 at sites of DNA damage induced by a UVA laser as well as UVC light.

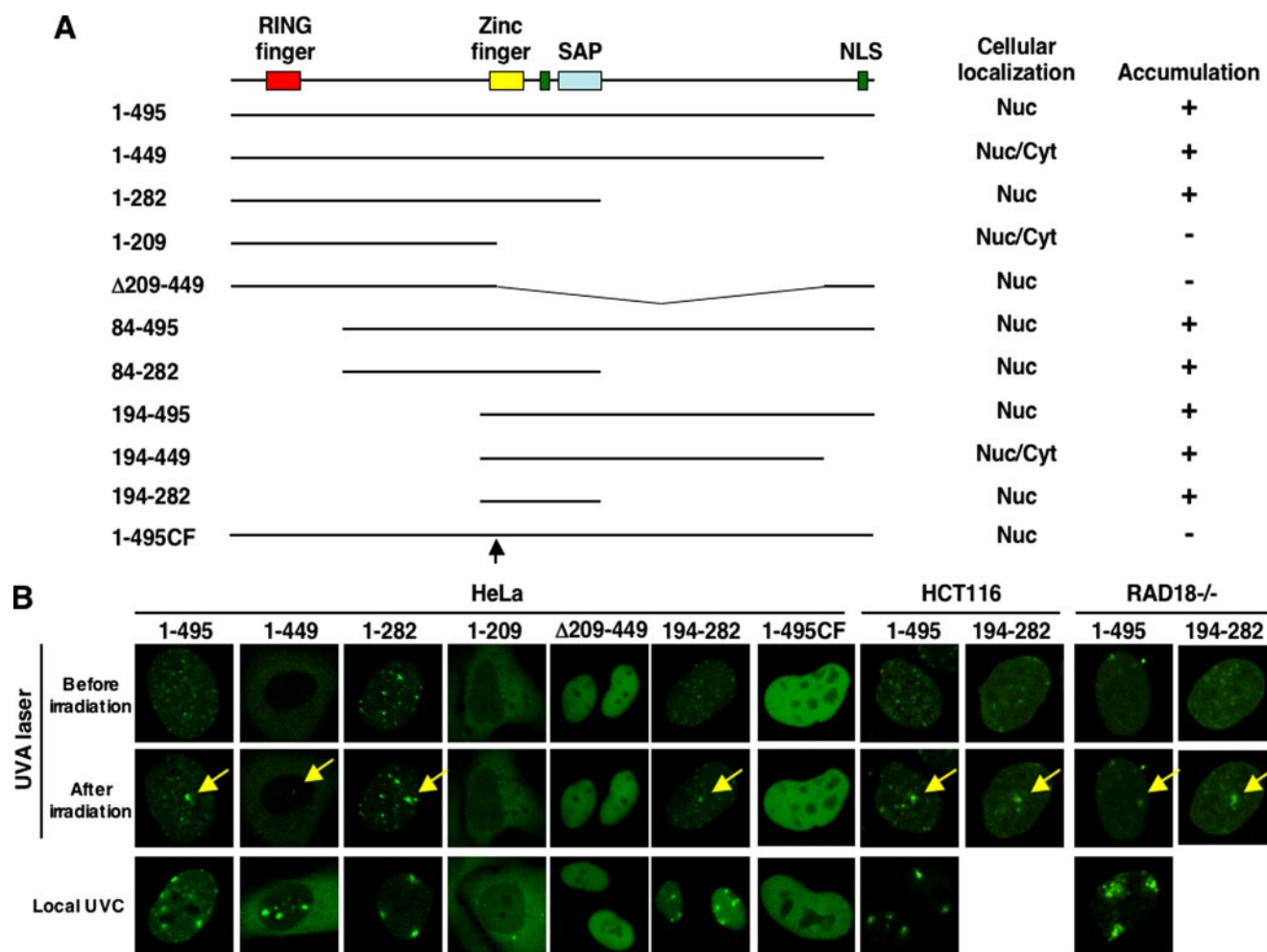
**The SAP Domain of RAD18 Is Required for Assembly of RAD18 at Stalled Replication Forks**—RAD18 mono-ubiquitinates PCNA at stalled replication forks, and this modification of PCNA leads to polymerase switching from replicative to lesion bypass polymerase such as Pol $\eta$  (39). Focus formation of Pol $\eta$  depends on RAD18 after UVC irradiation (28, 29), suggesting that recruitment of RAD18 at the site of stalled replication forks is necessary for translesion synthesis by Pol $\eta$ . To investigate whether the zinc finger motif is required for assembly of RAD18 at stalled replication forks, we transiently co-expressed GFP-tagged Pol $\eta$  and DsRed-tagged RAD18 in RAD18-deficient cells and examined Pol $\eta$  focus formation after UVC irradiation. Expression of the DsRed vector alone did not complement Pol $\eta$  focus formation in RAD18-deficient cells (not shown), while expression of DsRed fused to WT RAD18 clearly complemented Pol $\eta$  focus formation (Fig. 5). Although RAD18 1–495CF did not accumulate at sites of DNA damage induced

by both UVA laser and UVC light (Fig. 5B), expression of RAD18-(1–495CF) complemented Pol $\eta$  focus formation in RAD18-deficient cells (Fig. 5C). These data suggested that the zinc finger domain of RAD18 was not responsible for assembly at sites of stalled replication forks. In addition to the zinc finger domain, the middle part of RAD18 also contains a SAP domain (Fig. 5A), which is a putative DNA binding or protein-protein interaction domain (16, 40). To determine whether the SAP domain is required for assembly of RAD18 at stalled replication forks, we constructed deletion mutants of the SAP domain. RAD18-( $\Delta$ 248–284) but not RAD18-( $\Delta$ 248–284CF) accumulated at sites of DNA damage induced by both UVA laser and UVC light (Fig. 5B), indicating that the SAP domain is not required for accumulation of RAD18 at the site of DNA damage. Although RAD18-( $\Delta$ 248–284) could accumulate at the sites of DNA damage, expression of RAD18-( $\Delta$ 248–284) did not complement Pol $\eta$  focus formation in RAD18-deficient cells (Fig. 5C). Thus, the middle part of RAD18 is a damage response domain, while the zinc finger motif is responsible for accumulation at the site of DNA damage, independent of replication. The SAP domain is responsible for assembly at stalled replication forks.

## DISCUSSION

Replication-coupled DNA damage response mechanisms facilitate tolerance of DNA damage during replication to overcome termination of replication as well as to reduce the mutation rate at sites of DNA damage. The first step in the replica-





**FIGURE 4. Identification of the minimal domain of RAD18 for accumulation at damage sites.** *A*, schematic presentation of domains in RAD18 and the GFP-tagged mutant constructs used for accumulation assays are shown. Cellular distribution (Nuc, nucleus; Cyt, cytoplasm) of the mutants and accumulation (+, accumulated; -, not accumulated) at the sites of DNA damage are summarized on the right. *B*, HeLa cells were transfected with a series of GFP-tagged RAD18 deletion mutants. The accumulation of the GFP-tagged proteins at sites of damage induced by UVA laser irradiation or local UVC light was examined. HCT116 and RAD18-deficient cells were transfected with GFP-tagged full-length or deletion mutant (RAD18-(194–282)) and irradiated with UVA laser or local UVC light to determine the accumulation of the GFP-tagged proteins at the sites of damage. *Upper panel*, before irradiation; *middle panel*, 10 min after irradiation with UVA laser, *lower panel*, 1 h after irradiation at 100 J/m<sup>2</sup> UVC light with a porous filter.

tion-coupled response to DNA damage is the recognition of DNA damage or stalled replication forks by protein(s) involved in the mechanisms. RAD18 may be one of the proteins recruited immediately to the replication fork block, because mono-ubiquitination of PCNA by RAD18 and RAD6 is the first step initiating polymerase switches for translesion synthesis. However, the mechanisms for the recruitment of RAD18 at the site of the blocked replication fork are not yet known. To elucidate the mechanisms, we analyzed here the properties of human RAD18 in response to UV light as well as laser-induced DNA damage in living cells.

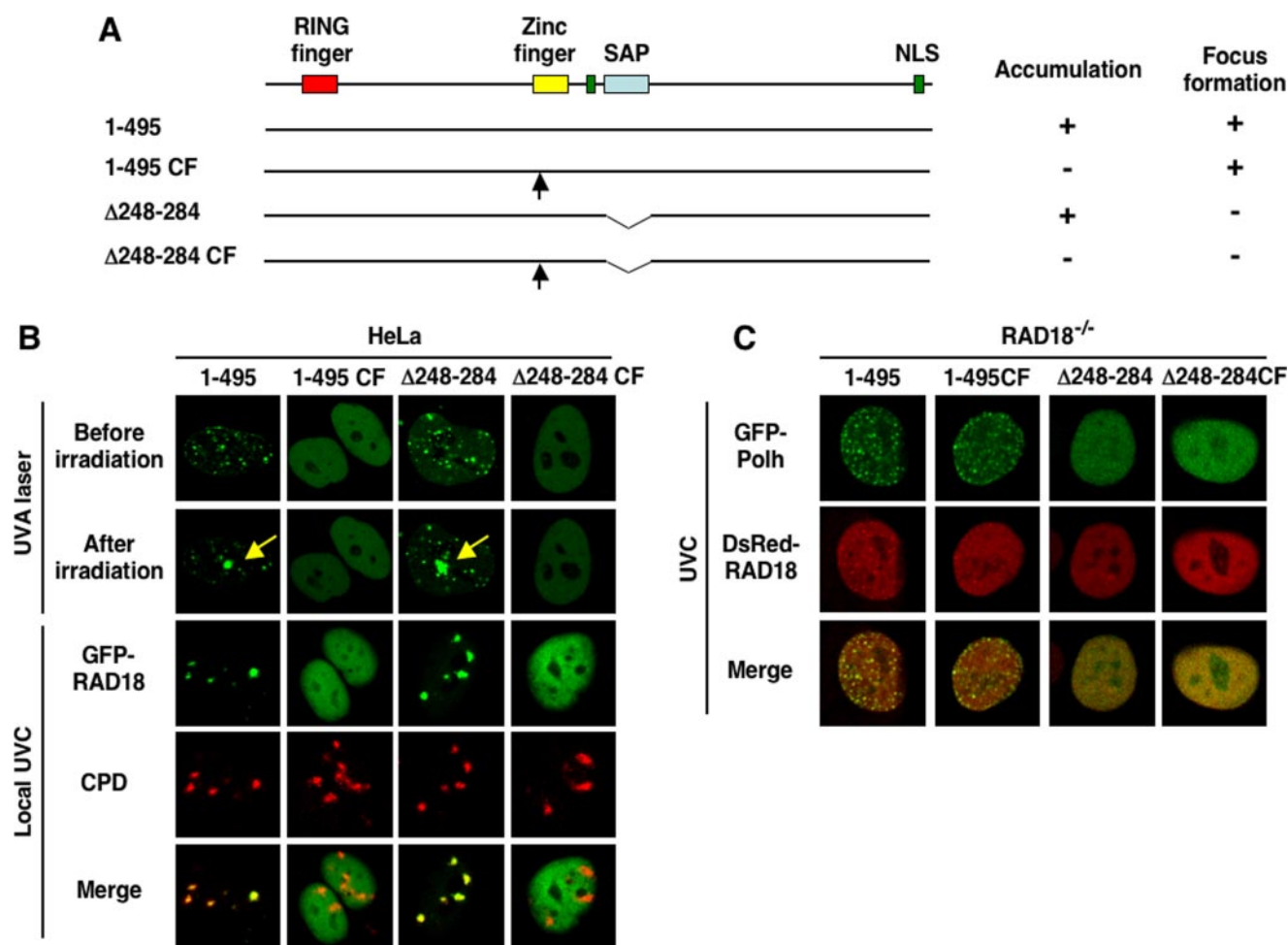
As shown in Fig. 1, RAD18 accumulates immediately at UVC light irradiated sites and remains for at least 8 h. Because RAD18 accumulates at the site of DNA damage induced by UVC light in XP-A cells (Fig. 1C), the accumulation is not affected by a functional NER complex, suggesting that the accumulation of RAD18 is independent of the repair of UVC light-induced lesions. Furthermore, RAD18 accumulates quite rapidly at the site of DNA damage, almost exclusively SSB, induced by UVA laser light (Fig. 2) This accumulation is influenced nei-

ther by a potent inhibitor of PARP activation nor by the absence of XRCC1 (not shown), suggesting that the accumulation of RAD18 at SSB is independent of the repair of SSB. These results suggest that accumulation of RAD18 is triggered by DNA damage itself or by circumstances induced by DNA damage but not by DNA repair processes or repair intermediates.

Because RAD18 plays an important role in PRR, the function of RAD18 is believed to be associated with DNA replication. However, we showed that RAD18 accumulated at DNA damaged sites even when DNA replication was inhibited (Fig. 3C), suggesting that RAD18 has a function not coupled with DNA replication. It has recently been reported that, like RAD18, REV1, another member of PRR, also accumulates at sites of DNA damage outside S phase (41), suggesting that PRR has a function that is not associated with DNA replication.

This replication-independent accumulation depends on the zinc finger motif of RAD18. Expression of RAD18 mutated in the zinc finger motif restored sensitivity to some of the DNA-damaging agents to levels seen with expression of WT RAD18 in RAD18-deficient cells (37), indicating that the zinc finger-

## Accumulation of RAD18 at Sites of DNA Damage



**FIGURE 5. The SAP domain of RAD18 is required for recruitment of RAD18 to the sites of stalled replication forks.** *A*, schematic presentation of domains in RAD18 and the GFP- or DsRed-tagged mutant constructs used for accumulation and complementation assays are shown. Accumulation (+, accumulated; -, not accumulated) of the mutant proteins at sites of DNA damage and the ability to complement Pol $\eta$  focus formation are summarized on the right. *B*, HeLa cells were transfected with GFP-tagged RAD18 mutants, with the SAP domain deleted and/or with a mutated zinc finger motif. The accumulation of the GFP-tagged proteins at the sites of damage induced by UVA laser irradiation (upper two panels) or local UVC light (lower three panels) was examined. *C*, assay for complementation of Pol $\eta$  focus formation by expressing RAD18 mutants in RAD18-deficient cells. RAD18-deficient cells were co-transfected with GFP-tagged Pol $\eta$  and DsRed-tagged RAD18, and irradiated with 20 J/m<sup>2</sup> UVC light without a porous filter. After 6 h of incubation, cells were fixed with 3.7% formaldehyde for 1 h and examined for Pol $\eta$  focus formation.

dependent function of RAD18 does not participate in resistance to the DNA-damaging agents tested. The zinc finger motif of RAD18, is a C2HC-type, an atypical and rare type of zinc finger (42). In contrast to the majority of classical zinc fingers, the C2HC-type zinc finger is unlikely to be involved in DNA contact. The zinc finger motif of RAD18 is homologous to that of human WHIP, which interacts with the N-terminal portion of Werner protein and plays a role in maintaining genomic stability (43, 44). In yeast simultaneous disruption of RAD18 and MGS1, the yeast homolog of WHIP, causes severe growth defect (45). Although the *rad18 mgs1* double mutant is almost a synthetic lethal, *mgs1* mutants are not sensitive to UV light and MMS, and only show an elevated rate of mitotic recombination (45). This suggests that RAD18 and MGS1/WHIP participate in damage tolerance against the same substrate. Therefore, the zinc finger-dependent function of RAD18 may be redundant with that of MGS1.

In contrast to the zinc finger motif, the SAP (SAF-A/B, Acinus, and PIAS) motif turned out to be important for Pol $\eta$  focus formation. The SAP motif consists of 35 conserved

amino acids forming two  $\alpha$ -helices and this motif has been found in chromatin proteins including scaffold attachment factors SAF-A and SAF-B, the transcription repressor PIAS, the abundant chromatin protein DEK and DNA repair proteins Ku70 and plant's poly(ADP-ribose) polymerase (16, 46). A common feature of these proteins is their ability to bind to chromatin and DNA (47). Because the polymerase activity of Pol $\eta$  is not necessary for its focus formation (48), the SAP motif of RAD18 may play an important role in recruiting Pol $\eta$  to stalled replication forks. The SAP motif is not only a DNA binding motif (16), but it also mediates protein-protein interaction. For example, PIAS1 (protein inhibitor of activated STAT1) has a SAP motif in the N terminus, and this domain is capable of binding to A/T-rich DNA fragments and p53 (40). It is, therefore, tempting to think that RAD18 may assemble to sites of stalled replication forks via a SAP motif-mediated interaction with chromatin, DNA or other proteins. Further studies, especially identification of the protein that interacts with distinct domains of RAD18 at the site of DNA damage, may elucidate the mechanisms by



which RAD18 responds to DNA damage and plays a role in the early steps of PRR in human cells.

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## Replication-dependent and -independent Responses of RAD18 to DNA Damage in Human Cells

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