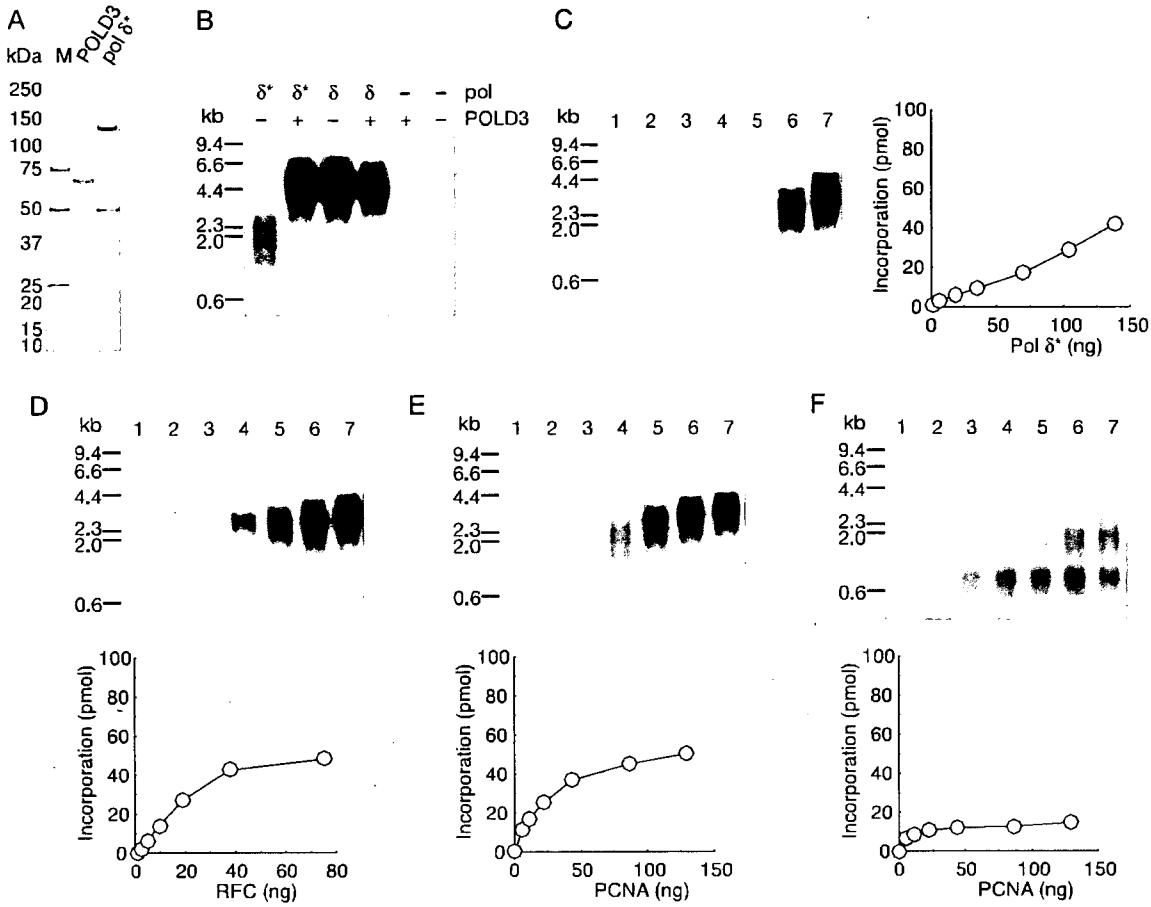

Figure 6. Dynamics of replication factors in the elongation phase of DNA synthesis with pol δ*. (**A**) SDS–PAGE analysis of purified recombinant proteins. Pol δ* (1.9 μg) and POLD3 (0.5 μg) were loaded on a SDS 4–20% gradient polyacrylamide gel and stained with CBB. (**B**) Reconstitution of pol δ with POLD3 and pol δ*. Reactions were carried out for 10 min under the conditions described in the Materials and Methods section except for pol δ* (70 ng) or pol δ (90 ng) in the absence (–) or presence (+) of POLD3 (20 ng). (**C–E**) Titration of pol δ* (C), RFC (D) and PCNA (E). Amounts of pol δ* were 0 ng (lane 1), 4.3 ng (lane 2), 17 ng (lane 3), 35 ng (lane 4), 70 ng (lane 5), 100 ng (lane 6), and 140 ng (lane 7). Amounts of RFC used in the titration were 0 ng (lane 1), 2.3 ng (lane 2), 4.7 ng, (lane 3), 9.4 ng (lane 4), 19 ng (lane 5), 38 ng (lane 6) and 75 ng (lane 7). Amounts of PCNA used in titration were 0 ng (lane 1), 5.4 ng (lane 2), 11 ng (lane 3), 22 ng (lane 4), 43 ng (lane 5), 86 ng (lane 6) and 129 ng (lane 7). (**F**) Titration of PCNA in the presence of HincII. Amount of PCNA is same as (E). Reactions in (C) were carried out for 10 min under the conditions described in the Materials and Methods section. Reactions in (D–F) were carried out for 10 min under the conditions described in the Materials and Methods section except for the amount of pol δ* (140 ng). Products were analyzed by 0.7% alkaline-agarose gel electrophoresis and incorporation of dNMP were measured as described.

reaction with pol δ* to only the decreasing affinity between PCNA and pol δ*. We therefore considered the possibility that loading and unloading of PCNA is equilibrated in both pol δ and pol δ* cases, and importantly, could be accelerated in the reaction with pol δ*.

DISCUSSION

Generally, proteins act during replication in two distinct modes, processively or distributively (32). The studies documented here showed very different dynamics of the various protein factors in the elongation phase of DNA replication.

DNA synthesis *in vitro* by pol δ has been investigated extensively. Previous studies have shown that pol δ itself is a very distributive enzyme, which turns into a processive polymerase when bound to the clamp, PCNA (11,12,15–17). However, even in the presence of PCNA, pol δ replicated M13 ss DNA through a number of dissociating and reassociating steps, as proposed for mammalian pol δ isolated from natural sources and overproduced in insect cells (34–36). Our results support the conclusion that human pol δ has a distributive nature for DNA replication in our model system *in vitro*.

The length of products synthesized by pol δ does not depend on the concentration of RFC (6,37). The processive nature of RFC might be explained in two alternative ways. One is that the sole role is loading of

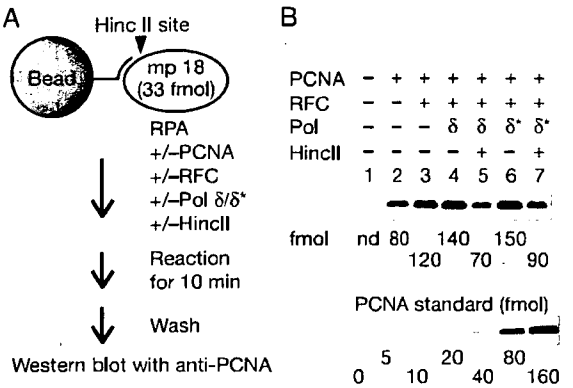

Figure 7. Amounts of PCNA loaded on DNA during elongation. (**A**) Outline of the assay. DNA was attached to magnetic beads via biotin–streptavidin linkage. The reactions were carried out for 10 min under the conditions described in the Materials and Methods section except for the amounts of pol δ (33 ng) and pol δ* (140 ng). (**B**) Western analysis. Chemiluminescence signals were detected with a CCD camera and quantified with reference to a standard curve for PCNA in the same blot.

PCNA at only the initiation step (35,38). Some biochemical data for yeast and human RFC support this possibility, because RFC has been found to dissociate from DNA after loading PCNA (38,47,48). The other explanation is that once RFC finds a 3′-hydroxyl end and loads PCNA, it then travels with PCNA and pol δ (39,40). In earlier work with yeast RFC, formation of tertiary complexes on DNA was also suggested (49). Later, Yuzhakov and colleagues (26) also reported that human RFC travels with pol δ, and a similar complex has been isolated from the elongation phase of SV40 replication (50). Our observations also provide support for continuous binding of RFC.

Because PCNA is a sliding clamp, it should be able to freely slide along double-stranded DNA and fall off at the ends (43,44,51). However, the fate of PCNA in the elongation phase of DNA replication is currently obscure. We here obtained evidence that some PCNA does not remain at the primer terminus after dissociation of pol δ. However, in titration experiments of PCNA, the longer products (around 4 kb) still remained even at low concentrations of PCNA, independent of the presence or absence of HincII (Figures 2E and G, 3E and G), indicating that significant fractions of the intermediates of elongation phase could continue DNA synthesis without supply of PCNA from solution. In such fractions, PCNA must remain at the primer terminus even after dissociation of pol δ. The observations support the conclusion that PCNA has a partially distributive nature in our DNA replication system with RFC *in vitro*. On the other hand, PCNA was entirely distributive in the RFC-independent reaction (Figure 4C). Taken the results together, we suggest the possibility that RFC could hold PCNA from which pol δ has detached.

We here propose a model for dynamics of replication factors during a dissociation–association cycle of pol δ (Figure 8). The elongation complex consists of RFC, PCNA and pol δ (Figure 8, stage I). Pol δ dissociates frequently from growing 3′-hydroxyl ends and PCNA during elongation of DNA replication (Figure 8, pathway 1). Then, PCNA from which pol δ has detached would be held by the remaining RFC (Figure 8, stage II). If pol δ reassociated quickly (Figure 8, pathway 2), RFC could remain around the primer terminus, and travel with pol δ and PCNA during elongation of DNA replication. In this cycle, PCNA is not released out of the replication complex, which implies 'recycling PCNA' (Figure 8). Since the DNA–RFC–PCNA complex (stage II) is not stable (52), the RFC could unload PCNA, or release the PCNA out of the primer terminus (Figure 8, pathway 3). Probably, the unloading reaction does not predominate, as shown in yeast RFC (53). If PCNA were available from

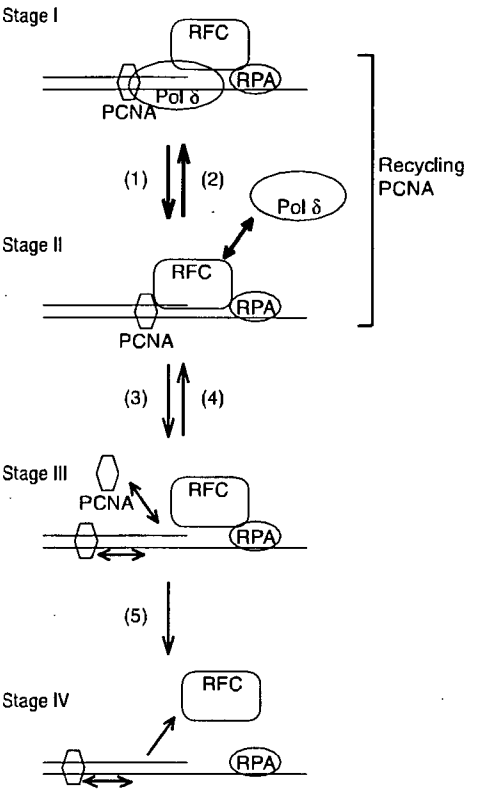

Figure 8. A model for dynamics of replication factors during pol δ dissociation–association cycles. The elongation complex consists of RFC, PCNA and pol δ in the elongation phase of DNA replication (Stage I). Pol δ contacts with PCNA and prevents RFC from dissociating. Contribution of RFC–RPA interaction for stable association in the complex has been proposed (26). Pathway 1, dissociation of pol δ leaving RFC and PCNA on DNA (Stage II). DNA–RFC–PCNA complex formation could be coupled with dissociation of pol δ, mediated by the POLD3 subunit. Pathway 2, reassociation of pol δ to form the elongation complex. The POLD3 subunit of pol δ might mediate efficient transfer of PCNA from RFC to pol δ. Pathway 3, unloading or sliding of PCNA out of the primer terminus, leaving RFC (Stage III). RFC probably interacts with RPA for retaining around primer terminus (26). Pathway 4, reloading of PCNA from solution or PCNA sliding back along the DNA to reform the DNA–RFC–PCNA complex. Pathway 5, dissociation of RFC from DNA (Stage IV). The main pathways are shown as thick arrows.

solution or along the DNA, RFC could incorporate PCNA into the complex (Figure 8, pathway 4). Otherwise, RFC would dissociate from the primer terminus (Figure 8, pathway 5). At higher concentrations greater than saturated amounts of RFC, the size of products was increased (Figures 2D and F, 3D and F). Probably, with such concentrations, RFC is sufficient for initiation and remaining RFC in solution could help to overcome the pausing site, implying dissociation of RFC at strong pausing sites. However, this was negligible under normal replication conditions, and was detectable in reactions omitting pol δ, as shown in lane 6 of Figure 5B. This suggested that pol δ prevents dissociation of RFC. In intensive studies of PCNA-loading reactions with yeast RFC, no stable RFC–DNA complex was detected in the absence of PCNA and only became detectable in the presence of ATPγS, and RFC dissociated quickly from DNA after loading PCNA (47,48). Therefore, it has been considered that RFC is absent in elongation complexes. Our results may explain the discrepancy regarding the prevention of dissociation of RFC by sequential loading of PCNA (pathway 4) and pol δ (pathway 2) in the replication assay, but not PCNA loading assays. Therefore, we consider that our model is consistent with the previous observations (38,47,48). Gomes *et al.* (47) has also proposed a loading pathway, in which PCNA–RFC complex first forms an ATP-dependent ring-opened complex and subsequently associates with DNA and delivers PCNA to the template–primer junction. RFC associated with the DNA cannot recruit PCNA nor load it at termini, first having to dissociate coupled with ATP hydrolysis (47). Our model is consistent with the loading mechanism. RFC is probably detached from the primer terminus before loading PCNA, but associated around primer terminus via interaction with RPA (stage III) as proposed previously (26).

Here, we further examined the dynamics of protein factors in the reaction with pol δ* to elucidate functions of the POLD3 subunit. This, together with its budding and fission yeast counterparts, Pol32 and Cdc27, respectively, has a PCNA-binding domain that is responsible for processive DNA synthesis on M13 ss DNA (54–57). We demonstrated that the size of products varies depending on the concentration of PCNA exhibiting an entirely distributive nature (Figure 6E). If PCNA were accumulated on the DNA reflecting an increase amount in solution, the requirement of large excess of PCNA in solution could be simply due to decreasing affinity between pol δ* and PCNA. We failed to demonstrate excessive accumulation of PCNA on the DNA (Figure 7B). Rather, the concentration of PCNA in solution little affected that on DNA. Therefore, it is impossible to attribute the entire distributive nature of PCNA on the reaction with pol δ* to simply a decreasing affinity between PCNA and pol δ*. We consider that the defect with pol δ* could be due to not only decreased affinity to PCNA, but also failure in recycling of PCNA after dissociation. In the reactions with pol δ, significant fractions of intermediates in the elongation stage could continue DNA synthesis without supply of PCNA from solution (Figure 2E), indicating efficient recycling of PCNA (Figure 8, pathways 1 and 2). In contrast, such a fraction was not detected in reactions with pol δ* (Figure 6E), presumably due to predominant loss of PCNA from the primer terminus. This could be a consequence of unloading of PCNA by RFC, since excessive accumulation of PCNA was not observed (Figure 7B) under conditions whereby excess loading of PCNA from solution was expected (Figure 6E). Yeast studies have predicted a second function of subunits enhancing processivity of pol δ in a manner independent of the PCNA-binding site (56). We propose that the previously unknown function of POLD3 subunit is stimulation of recycling of PCNA in the dissociation–association cycle with pol δ.

It has been shown that RFC binds non-specifically to DNA with a potential for loading PCNA on the double-stranded DNA, independent of the primer ends (58). Is the processivity of RFC observed in this work only apparent and due to non-specific loading of PCNA? If PCNA molecules were loaded anywhere on the double-stranded DNA and accumulated on the DNA, it could explain that size of the products of DNA synthesis depends on the concentration of PCNA, especially in reactions with pol δ* (Figure 6E). However, we failed to detect excessive accumulation of PCNA on the DNA (Figure 7B). Furthermore, spontaneously loaded PCNA from the end of DNA did not well support elongation, even an excessive amount of PCNA was present in the RFC-independent reactions (Figure 4), suggesting that loading of PCNA at primer terminus is crucial for the efficiency. We speculate that PCNA loaded non-specifically on the DNA is probably ineffective, just like spontaneously loaded PCNA. Although we could not exclude the possibility of contribution of non-specific loading of PCNA to the apparent processivity of RFC, it is probably not predominant in our reaction conditions.

PCNA functions as a platform not only for elongation but also for Okazaki fragment processing through interaction with protein factors, FEN1 and DNA ligase I. Recently, the Kunkel laboratory has provided evidence that pol ε is active in DNA synthesis on the leading strand (59), suggesting the pol δ is active on the lagging strand (59,60). Consistent with this, stable association of RFC in elongation complexes with pol δ for efficient utilization of PCNA could thus be of benefit to maturation of Okazaki fragments. Consistently, physical interactions between RFC and DNA ligase I have been demonstrated (61), implying a functional significance of stable association of RFC in elongation complexes on the lagging strand.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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DNA damage induced ubiquitylation of RFC2 subunit of RFC complex

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Running title: RPA-sensitive ubiquitylation of RFC2

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Many proteins involved in DNA replication and repair undergo post-translational modifications such as phosphorylation and ubiquitylation. PCNA (Proliferating Cell Nuclear Antigen, a homo-trimeric protein that encircles double-stranded DNA to function as a sliding clamp for DNA polymerases) is mono-ubiquitylated by the RAD6-RAD18 complex, and further poly-ubiquitylated by the RAD5-MMS2-UBC13 complex, in response to various DNA-damaging agents. PCNA mono- and poly-ubiquitylation activate an error-prone translesion synthesis pathway and an error-free pathway of damage avoidance, respectively. Here, we show that RFC (Replication Factor C, a hetero-pentameric protein complex that loads PCNA onto DNA) is also ubiquitylated in a RAD18-dependent manner in cells treated with alkylating agents or H_2O_2. A mutant form of RFC2 with a D228A substitution (corresponding to a yeast Rfc4 mutation that reduces an interaction with RPA, a

ssDNA-binding protein) is heavily ubiquitylated in cells, even in the absence of DNA damage. Furthermore, RFC2 was ubiquitylated by the RAD6-RAD18 complex *in vitro* and its modification was inhibited in the presence of RPA. The inhibitory effect of RPA on RFC2 ubiquitylation was relatively specific since RAD6-RAD18-mediated ubiquitylation of PCNA was RPA-insensitive. Our findings suggest that RPA plays a regulatory role in DNA damage responses via repression of RFC2 ubiquitylation in human cells.

Cellular DNA is continuously damaged by a vast variety of endogenous and exogenous genotoxicants. When genomic DNA is damaged, cells respond by activation of complex signaling network that delay cell-cycle progression, induce repair of lesions, activate damage tolerance pathways and trigger apoptosis or senescence (1,2). It is hypothesized that DNA damage-inducible signaling pathways serve important tumor-suppressive roles and prevent mutations that could lead to malignancy. Various genotoxins elicit different forms of DNA damage and result in distinct signal transduction pathways and biological outcomes. Distal steps of DNA damage-induced checkpoint signaling pathways that result in inhibition of the cell cycle are relatively well understood (3,4). However, molecular details of proximal signaling events and lesion-specific DNA damage recognition events are less clear.

DNA replication and repair requires the coordinated actions of multiple proteins on small regions of DNA. A limited number of proteins serve to coordinate multiple replication and repair events. Some proteins function commonly in DNA replication and repair, and frequently have a crucial role in both processes. Three such examples are replication protein A (RPA), proliferating cell nuclear antigen (PCNA) and replication factor C (RFC). RPA was originally identified as a eukaryotic single-stranded DNA binding protein essential for *in vitro* replication of SV40 DNA (5,6). PCNA is a trimer of three identical subunits arranged head-to-tail to generate a ring-like structure with a large central cavity for encircling DNA. It is well established that PCNA provides a mobile platform to serve as anchor and processivity factor for DNA polymerases during chromosomal replication (7,8). PCNA is loaded onto the primer-template junction in an ATP-dependent manner by a multi-protein clamp loader, RFC (9,10). RFC binds preferentially to double-stranded/single stranded junctions with a recessed 3' end, which is the DNA target for PCNA loading.

RPA, PCNA and RFC are key proteins that play central roles in DNA replication, participating in competitive polymerase switching during lagging strand synthesis. The DNA polymerase -primase complex (Pol) that synthesizes an RNA-DNA hybrid primer

requires contact with RPA to remain stably attached to the primed site. For processive DNA synthesis to follow, Pol must be replaced by DNA polymerase (Pol). Replacement of Pol by Pol is initiated by interactions between RFC and RPA, which disrupt Pol -RPA interactions and result in removal of Pol from DNA. After RFC loads PCNA onto the primed site, Pol associates with PCNA by displacing RFC. The switching process is indeed coordinated by RPA, via cooperative interactions with PCNA and RFC (11,12). RPA, RFC, and PCNA also play key roles in DNA repair by interacting with many DNA repair enzymes (13-15). Such interactions are believed to play roles in DNA damage recognition and in recruiting and positioning of DNA repair enzymes.

RFC consists of five different subunits, which are homologous to one another and are members of the AAA+ family of ATPases (16,17). The RFC1(p140) subunit is sometimes referred to as 'large subunit', as it contains both N- and C-terminal extensions beyond its region of homology with the four 'small' subunits. The four small RFC subunits are designated RFC2(p40), RFC3(p36), RFC4(p37) and RFC5(p38) in mammals. Three protein complexes with resemblance to RFC have been recently described, which are involved in maintaining genome stability. These RFC-like complexes (RLCs) share four common small subunits (RFC2-5) and each carry a unique large subunit (RAD17, CTF18 or ELG1) replacing the RFC1. These RLCs are involved in the checkpoint response (RAD17-RFC), sister chromatid cohesion (CTF18-RFC) and maintenance of genome stability (ELG1-RFC) (18,19).

DNA-damage sensors and repair proteins must react in a rapid and efficient manner to execute their functions. Frequently, the regulation of these proteins involves post-translational modifications, such as phosphorylation and ubiquitylation, to help modulate the assembly and disassembly of complexes, and to assist targeting and the regulation of enzymatic activity in a timely manner. For example, RPA is hyper-phosphorylated upon DNA damage or replication stress by several checkpoint kinases (20). Hyperphosphorylation alters RPA-DNA and RPA-protein interactions (15,21). Recent studies in the DNA-repair field have highlighted the expanding role of ubiquitylation in the regulation of diverse DNA-repair processes and pathways. One of the most striking examples of how ubiquitylation can affect protein function is that of PCNA in budding yeast *Saccharomyces cerevisiae*. Following DNA damage, PCNA can be mono-ubiquitylated, or poly-ubiquitylated on the K164 residue and each modification results in a different outcome with respect to DNA synthesis and repair (22,23). Mono-ubiquitylated PCNA directs translesion synthesis (TLS) via error-prone DNA

polymerases, while poly-ubiquitylated PCNA is associated with an error-free DNA repair pathway (22,23). Mammalian PCNA also undergoes mono-ubiquitylation after UV irradiation, and mono-ubiquitylated PCNA preferentially binds to TLS polymerases that contain one or two copies of ubiquitin-binding domains (24-27).

In contrast to RPA and PCNA, damage-dependent modification of RFC has not been described. Recent studies have significantly broadened the scope of the role of ubiquitylation to include regulatory functions in DNA repair and damage response pathways. Therefore, in this report we investigated whether the clamp loader RFC is likewise subjected to regulated modification. We have examined the modification of all subunits in RFC and RLCs. We demonstrate that RFC2 and RFC4 are ubiquitylated following treatment of cells with alkylating agents. The ubiquitylation is partially dependent on RAD18. Surprisingly, RPA inhibits the RAD18-dependent ubiquitylation of RFC2. Our results suggest that RFC regulates the DNA damage response pathway via interaction with RPA and ubiquitylation.

Experimental Procedures

Plasmid constructs- To generate pCDNA.RFC2(p40)Flag and pCDNA.RFC2(p40)HA, human p40 coding region was amplified by PCR as a EcoRI-XhoI fragment. The PCR product was inserted into the EcoRI-XhoI site either of pCDNA-C-Flag or pCDNA-C-HA. To generate pCAGGS.RFC2(p40), the human p40 coding region was amplified by PCR as a SalI-XhoI fragment. The PCR product was inserted into the XhoI site of pCAGGS. pCDNA-C-Flag and pCDNA-C-HA was constructed by inserting the Flag or HA epitope into the XhoI-XbaI site of pCDNA3.1. Expression plasmids containing human RFC1-FLAG, human FLAG-RAD17, human FLAG-CTF18, human FLAG-p38, human FLAG-p37, human FLAG-p36 were constructed by inserting their cDNA described in (28) into pCDNA3. Although N-terminal and C-terminal tagged forms of each RFC2 subunit were used, the presence of the epitope-tag did not affect RFC2 regulation at least in the context of experiments reported in this study. pCAGGS.Flag-Ubiquitin and pCAGGS.hRAD18 were constructed as previously described (25). The expression plasmids for human RFC, PCNA were described earlier (29,30) and that for human RPA, p11d-tRPA (31), was a generous gift of Dr. Marc S. Wold (University of Iowa College of Medicine, Iowa City, Iowa). Mouse E1 expression vector RLC(32,33) was a generous gift of Dr. Hideyo Yasuda (School of Life Science, Tokyo University of Pharmacy, and Life Science, Tokyo, Japan). Human cDNAs for RAD6A and RAD18 amplified from a HeLa cDNA library by PCR introducing a NdeI site at

the start codon were cloned together into pET20b(+) (Novagen) as an artificial operon. After cloning the PCR fragments, the nucleotide sequences were verified. All the expression plasmids of PCNA, RPA, RFC, E1, RAD6A and RAD18 were designed for production of intact proteins, without any affinity tags.

Cell culture and transfection- 293A and HCT116 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS). HCT116 *RAD18*$^{-/-}$cells were established as described previously (25). Cells were transfected with Lipofectamine Plus (Invitrogen) or Lipofectamine 2000 according to the manufacture's protocol. 2.4 μg of plasmid DNA was used to transfect each 6 cm plate of cells. Transfected cells were treated with genotoxins 24 hr post-transfection.

Genotoxin and Inhibitor Treatments- Asynchronous cell cultures were grown to ca. 80% confluency. For UV treatment, cells were washed with PBS, exposed to UV light (254 nm) at a fluence rate of 43 Jm^{-2}/s. For genotoxin and inhibitor treatment, hydroxyurea (HU, 1 M in H_2O), aphidicolin (dissolved in DMSO), methyl methanesulfonate (MMS, dissolved in DMSO), ethyl methanesulfonate (EMS, dissolved in DMSO), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, dissolved in DMSO), H_2O_2 (diluted in PBS), mitomycin C (MMC), bleomycin (Bleo, dissolved in H_2O) or camptothecin (CPT, dissolved in DMSO), was added to the culture media to give a final concentration of 2 mM, 0.025mM, 0.1-1.7 mM, 20 mM, 0.7 mM, 0.5 mM, 0.01mM, 0.05mg/ml or 20 nM, respectively and cells exposed for 8 hr unless otherwise stated.

Antibodies- A mouse monoclonal antibody against *Drosophila* RFC40 (anti-dRFC40) was used for probing human RFC2(p40). A hybridoma cell line producing anti-dRFC40 antibody is a kind gift from Dr. Gerald M. Rubin (University of California, Berkeley) and monoclonal antibody was purified as described previously (34). To test whether anti-dRFC40 antibody cross-reacts with human RFC2(p40), an HA epitope-tagged form of hRFC2(p40) was over-expressed in 293A cells by transfection, cell lysate was recovered 24 hr post-transfection, and then immunoblotted with either anti-dRFC40 or anti-HA antibody. An anti-dRFC40-reactive protein band migrating at 40 kDa was clearly observed only in extracts from HA-hRFC2(p40)-transfected cells and corresponded to the species detected with an anti-HA antibody (Supplementary Fig. 1). Therefore, the anti dRFC40 antibody recognizes human RFC2(p40). To avoid confusion we refer to the anti-dRFC40 antibody as 'anti-RFC2' antibody in this report.

Other commercial antibodies used in this study are: anti-HA (Y-11, Santa Cruz Biotechnology), anti-FLAG (M2, Sigma), anti-RFC1 (H-300, Santa Cruz Biotechnology), anti-RAD17 (H-3, Santa Cruz Biotechnology),

anti-Tubulin (B-5-1-2, Sigma), anti-Histone H3 (6.6.2, Upstate and ab1791, Abcam) and anti-PCNA (PC10, Oncogene).

Preparation of Cell Lysate and Chromatin Fraction- 293A cells in a 3.5 cm or 6 cm dish were washed twice with ice-cold PBS and then harvested into RIPA buffer (1xPBS, 1%NP-40, 0.5% sodium deoxycholate, 0.1%SDS, 1mM PMSF, 1mM sodium orthovanadate, and protease inhibitor (Nacalai)). The cell suspensions were incubated for 30 min on ice, and then the NP40-0.1%SDS-insoluble fraction and soluble fraction were separated by centrifugation. The soluble fraction was used as Supernatant (Sup) fraction. The resultant pellet was washed with PIPA buffer four times and then sonicated after adding SDS-PAGE loading buffer (7% Glycerol, 22% SDS, 50 mM Tris-HCl (pH6.8), 5% b-mercaptoethanol). The resultant solution was used as Chromatin fraction. We have confirmed few contaminations in each Sup and Chromatin fraction using anti-Tubulin and anti-Histone H3 antibodies (Supplementary Fig. 2).

SDS-PAGE and Western blotting- Cell extracts were resolved by electrophoresis on 7.5 or 10% SDS-PAGE gels. Following transfer onto PVDF or nitrocellulose filters, the blots were incubated with antibodies and immunoblots were visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia or DURA, Pierce), according to the manufacture's instructions.

Immunoprecipitation- Cell extracts were incubated with monoclonal mouse anti RFC2 (dRFC4(p40)) antibody for 1 hr at 4 °C, and then with 25 μl of A/G-agarose (Santa Cruz). After incubation for overnight at 4 °C, the beads were washed with PBS three times, boiled in Laemmli buffer for 5 min, and the bound proteins were analyzed by electrophoresis and immunoblotting.

Protein Purification- Human RFC, PCNA, RPA were purified as described (29,30). Mouse E1 was overproduced in insect cells and purified as described (35). Human RAD6A-RAD18 complex was overproduced in *E. coli* cells and then purified by column chromatography (phosphocellulose, heparin sepharose, MonoQ and gel filtration) from *E. coli* cell lysate. Protein concentrations were determined by Bio-Rad protein assay kit (Bio-Rad) using BSA as the standard. Bovine ubiquitin was purchased from Sigma.

in vitro ubiquitylation assay- The reaction mixture (25 μl) contained 20 mM HEPES-NaOH (pH 7.5), 50 mM NaCl, 0.2 mg/ml BSA, 1 mM DTT, 10 mM MgCl2, 1 mM ATP, 33 fmol of singly primed single stranded M13 mp18 DNA (30) , 1.0 μg (9.1 pmol) of RPA, 86 ng (1.0 pmol as a trimer) of PCNA, 75 ng (260 fmol) of RFC, and 100 ng (850 fmol) of mouse E1, 175 ng (2.4 pmol) of RAD6A-RAD18 complex, and 12.5 μg (1460 pmol) of ubiquitin. After incubation at 30°C for 60 min, reactions were terminated with 2microl

of 300 mM EDTA.

Structural model building- Homology modeling of the human clamp-loader/clamp complex was performed using MODELLER v7.7 (36). The homologous structures were defined using the fold recognition server FORTE (37). The atomic coordinate of the clamp/clamp-loader complex (PDB:1SXJ) was selected as a templates for model building. Before submission to MODELLER, the sequence–structure alignment obtained from FORTE was used. Due to the lack of the template structure, the N-terminal 582 residues of human RFC1 were not modeled. The figures were prepared using MOLMOL (38). Coloring of each RFC subunits and PCNA was according to Figure 2 in the review (39).

RESULTS

Specific DNA damaging agents induce modification of RFC2. To analyze the modification of each subunit of the RFC complex, a Flag epitope-tagged form of each subunit of RFC and RLCs was expressed in human 293A cells. Transfected cells were treated with UV, -ray, hydroxyurea (HU) or MMS, and then cell extracts were prepared. The cell extracts were separated into NP40-insoluble chromatin fractions (CF) and soluble fractions (Sup). RFC and RLC subunits in each fraction were analyzed by SDS-PAGE and Western blotting (Fig. 1A). Following MMS treatment all of the subunits, except for CTF18 and RFC5, accumulated in the chromatin fraction, whereas no accumulation was observed following treatments with UV, -ray or HU. Levels of soluble CTF18 and RFC5 decreased after MMS treatment, although we did not detect concomitant increases in the chromatin-bound levels of these subunits (Fig. 1A). Taken together, the results of Fig. 1A demonstrate that the levels and subcellular distribution of RFC and RLC subunits are regulated in response to MMS.

It was important to determine whether endogenous RFC and RLC subunits were also redistributed to chromatin in response to MMS. Therefore, we determined the effects of MMS on endogenous RFC1, RAD17 or RFC2 proteins for which good antibodies are available. As shown in Fig. 1B, endogenous RFC1, RAD17 and RFC2 accumulated in the chromatin fraction of MMS-treated 293A cells. Similar to ectopically expressed tagged proteins, endogenous RFC subunits are redistributed to chromatin in response to MMS treatment.

Interestingly, we observed prominent forms of ectopically-expressed RFC2 and RFC4 that migrated with reduced electrophoretic mobility on SDS-PAGE gels in chromatin fractions from MMS-treated 293A cells (Fig. 1A, lane 7). Electrophoretically-retarded species of endogenous RFC2 were also evident in chromatin fractions of MMS-treated 293A cells (Fig. 1B, lane 7). The electrophoretically-shifted

form of RFC2 was more prominent than that of RFC4 (Fig. 1A). Therefore we focused on RFC2 and further analyzed its MMS-induced modification.

We performed quantitative analyses to determine the amount of chromatin-bound RFC2 relative to the soluble fraction in MMS-treated cells. In 293A cells ectopically expressing HA-tagged RFC2, more than 90% of the RFC2 accumulated to the chromatin fraction following 8 h of MMS treatment, whereas in untreated cells, less than 10% of RFC2 was present in the chromatin fraction (Supplementary Fig. 3). Following MMS treatment, we consistently detected two electrophoretically-retarded -RFC2-reactive proteins in the chromatin fraction. The apparent molecular mass of electrophoretically-retarded RFC2 is consistent with ubiquitylation. The two putative ubiquitylated forms of RFC2 (shown in Fig. 1) might correspond to species that are mono-ubiquitylated on different residues. However, we cannot exclude the possibility that modifications other than ubiquitin are also present on the shifted RFC2. Furthermore, smaller -RFC2-reactive proteins, possibly corresponding to degradation products, were detected in soluble and chromatin fractions from both control and MMS-treated cells (Fig. 1B and Supplementary Fig. 3).

The electrophoretically-retarded forms of RFC2 were induced by MMS in a dose-dependent manner (Fig. 1C). At lower concentrations of MMS (0.1 mM or 0.213 mM), no RFC2 band-shift was detectable. However, treatment with higher concentrations of MMS (0.425 mM, 0.85 mM or 1.7 mM) induced prominent electrophoretically-retarded forms of RFC2 on chromatin (Fig. 1C).

In the experiments described above, the cells were treated with MMS for 8 h. We subsequently examined the kinetics of RFC2 modification by treating 293A cells with MMS (0.85 mM) for 1 h and preparing samples for immunoblotting at 0, 2, 5 and 8 h following MMS-treatment. As shown in Fig. 1D, the shifted forms of RFC2 were detectable by 5 h after treatment of cells with MMS (lane 4). Similar to results of Fig. 1A, the genotoxin-induced RFC2 mobility shift was specific for MMS since UV irradiation (30 J/m^2; lanes 7-9) did not induce RFC modification at any time point tested (although as expected, UV induced PCNA mono-ubiquitylation under these experimental conditions). Conversely, little or no PCNA modification was detectable under the conditions used for the experiment shown in Fig. 1D (lanes 2-5), although low levels of PCNA ubiquitylation were observed when cells were treated with 0.85 mM of MMS for longer times (data not shown).

The results of Figs. 1A and 1D indicated that MMS-induced RFC2 modification is not a general response to DNA damage. To gain insight into the significance of RFC2 modification, 293A cells ectopically expressing

RFC2-HA were treated with a more extensive panel of DNA-damaging agents for 8 h and proteins in resulting chromatin fractions were analyzed by immunoblotting with the anti-RFC2 antibody (Fig. 1E, upper panel). DNA damaging agents we tested included alkylating agents (EMS and MNNG), an oxidizing agent (H_2O_2), a DNA crosslinking agent (MMC), double strand break inducing agents (Bleomycin and IR) and the topoisomerase I inhibitor camptothecin (CPT). Of the genotoxic agents tested, only EMS, MNNG and H_2O_2 induced the shifted RFC2 band evident in MMS-treated cells (Fig. 1E, upper panel, lanes 7-10). Many of the agents failing to induce the RFC2 bandshift nevertheless induced very robust PCNA mono-ubiquitylation (Fig. 1E, lower panel). Therefore, we conclude that RFC2 modification is a specific response to a subset of genotoxins.

RAD18-dependent ubiquitylation of human RFC2. To test whether the shifted RFC2-specific band in MMS-treated cells was due to ubiquitylation, RFC2-HA was co-expressed with Flag-tagged ubiquitin in 293A cells. The transfected cells were treated with MMS. Endogenous and HA-tagged RFC2 proteins were immunoprecipitated with anti-RFC2 antibody from cell lysates, and the precipitated proteins were immunoblotted with either anti-RFC2 (Fig. 2A upper panel) or anti-Flag antibody to detect Flag-ubiquitin-modified proteins (lower panel). Anti-RFC2-reactive bands migrating at the sizes expected for mono-ubiquitylated RFC2 (48 kDa) were observed in our anti-RFC2 immunoprecipitates (Ub-RFC2 in lanes 3 and 4). In addition to the 48 kDa Ub-RFC2 band, extra two slowly migrating bands (51 and 62 kDa) were observed in the immunoprecipitates obtained from cells transfected with Flag-tagged ubiquitin (Flag-Ub-RFC2 in lane 4), which were also detectable by immunoblotting with anti-Flag antibody (lane 8). From these results we conclude that the slow migrating forms of RFC2 in MMS-treated cells are ubiquitylated species.

In *S. cerevisiae* and human cells, mono-ubiquitylation of PCNA is dependent on the Rad18 E3-ubiquitin ligase (22,24,25). To determine whether ubiquitylation of RFC2 was similarly dependent on RAD18, RFC2 modification was tested in RAD18-overexpressing 293A cells (Fig. 2B) and *RAD18*-deficient HCT116 cells (Fig. 2C). As shown in Fig. 2B, over-expression of RAD18 induced the ubiquitylation of RFC2-HA and PCNA even in the absence of MMS treatment. Conversely, MMS-induced ubiquitylated forms of RFC2 decreased considerably (by 50%) in HCT116 $RAD18^{-/-}$ cells compared to those in matched HCT116 $RAD18^{+/+}$ cells (Fig. 2C). These results suggest that RFC2 mono-ubiquitylation in MMS-treated cells is mediated, at least in large part by RAD18, most probably as a complex with RAD6. Interestingly, RAD18-overexpression

also induced chromatin accumulation of RFC2 (Fig. 2B). Ubiquitylation and chromatin accumulation of RFC2 (and also RFC4) was observed in response to MMS treatment and Rad18-overexpression. Because MMS treatment induced chromatin accumulation of each RFC subunit (Fig. 1A), it is most likely that increased chromatin loading of the entire RFC complex occurs in response to MMS.

A RFC2 mutant is ubiquitylated in the absence of DNA damage. It has been reported that the RFC2(p40) subunit of human RFC binds the large subunit of RPA (11). In *S. cerevisiae*, a mutation in *rfc4* (yeast homolog of human *RFC2(p40)*) was found to display synthetic lethality with mutation in the gene encoding Rpa1 (the large subunit of *S. cerevisiae* RPA) (40). Interestingly, this mutant Rfc4(p40) showed weaker physical interaction with RPA than the wild type Rfc4(p40). This mutation, resulting in an amino acid change of aspartate to asparagine at residue 201, maps to the RFC box VIII, which is one of the conserved motifs found in all RFC subunits (16,41). The D201 residue of *S. cerevisiae* Rfc4 is conserved and found at an identical position in RFC2 from higher eukaryotes, including humans (Fig. 3A). We replaced D228 of human RFC2 (which corresponds to *S. cerevisiae* Rfc4 D201) with an asparagine residue (D228N) or an alanine (D228A). HA-tagged forms of mutant or wild-type RFC2 were expressed in 293A cells by transfection (Fig. 3B). The wild-type and mutant forms of RFC2-HA were expressed at similar levels; however, while the wild-type and D228N mutant RFC2 proteins showed no ubiquitylation of RFC2, the D228A mutant RFC2 protein underwent extensive modification without any genotoxin treatment (Fig. 3B, lane 8). The multiple shifted bands of RFC2 D228A decreased by 55% in HCT116 *RAD18*$^{-/-}$ cells compared to those in matched HCT116 *RAD18*$^{+/+}$ cells (Fig. 3C). Therefore, we conclude that the multiple Rad18-dependent species we observed correspond to mono- and poly-ubiquitylated forms of RFC2. As described in the previous sections, we observed mono-ubiquitylated forms of the wild-type RFC2-HA in MMS-treated cells, but did not observe high levels of its poly-ubiquitylated forms. The results of Fig. 3B indicate that the RFC2 D228A mutant is extensively ubiquitylated and accumulates as multiple poly-ubiquitylated species (even in the absence of genotoxin treatments) when ectopically expressed. Although the difference in susceptibility to spontaneous ubiquitylation between D228A and D228N is unexpected, by analogy with the *S. cerevisiae* Rfc4 D201N mutant protein, it is most likely that D228 of human RFC2 is also involved in interaction with RPA. While we have not formally verified the reduced interaction of human RFC D228A with RPA, we infer that RAD6-RAD18-mediated RFC2 ubiquitylation is regulated by interaction with RPA (see below).

RFC2 is modified by the RAD6-RAD18 complex in vitro. We subsequently examined whether RFC2 could be modified by the RAD6-RAD18 complex *in vitro*. Recombinant RFC complex, (including RFC1-5 proteins of human origin), was expressed in *E. coli* and then purified. Mono-ubiquitylation of RFC2 *in vitro* was investigated by mixing the RFC1-5 complex with purified recombinant RAD6A (E2 ubiquitin-conjugating enzyme)-RAD18 (E3 ubiquitin ligase) complex. As shown in Fig. 4, RFC2 was monoubiquitylated *in vitro* when incubated in the presence of purified RAD6A and RAD18 plus ubiquitin and its activating enzyme (lane 2), although at a much lower efficiency when compared with PCNA. It should also be noted that the *in vitro* modification of RFC2 generated only a single mono-ubiquitylated species while at least two mono-ubiquitylated forms of RFC2 (corresponding to mono-ubiquitylation at different residues) resulted from MMS treatment of intact cells. The reason for the differential patterns of RAD18-mediated RFC2 mono-ubiquitylation observed *in vitro* and in intact cells is not yet clear, but could result from the existence of additional RFC2-directed E3 ligases *in vivo*. The difference also indicates that *in vitro* assay conditions do not fully recapitulate the complexity of events involved in RPA-sensitive RFC2 ubiquitylation at stalled replication forks *in vivo*. It should be noted that our *in vitro* assay uses primed M13 ssDNA, which mimics the leading strand synthesis rather than the lagging strand synthesis that requires the RFC complex more frequently. PCNA did not affect RFC2 mono-ubiquitylation (lane 4), although the modification was dependent on the presence of DNA (data not shown). Interestingly, however, the addition of RPA inhibited RAD6-RAD18-dependent mono-ubiquitylation completely (lanes 3 and 5). In parallel reactions, RPA did not affect the mono-ubiquitylation of PCNA (lanes 4 and 5). Therefore, RPA specifically inhibits RAD18-dependent mono-ubiquitylation of RFC2. The inhibition of RAD18-mediated RFC2 ubiquitylation by RPA *in vitro* is consistent with our finding that the RFC2 D228A mutant is more extensively modified than wild-type RFC2 in intact cells.

DISCUSSION

Protein ubiquitylation is critical for numerous cellular functions, including DNA damage response pathway. In this report we have demonstrated that RFC2 is ubiquitylated in human cells via DNA damage-independent and genotoxin-inducible mechanisms. RFC2 ubiquitylation is partially dependent on RAD18 as demonstrated by the decreased MMS-induced RFC2 ubiquitylation in *RAD18*$^{-/-}$ cells compared to matched *RAD18*$^{+/+}$ HCT116 cells (Fig. 2C). Conversely, RFC2 undergoes genotoxin-independent mono-ubiquitylation in cells over-expressing RAD18.

RAD18-dependent mono-ubiquitylation of RFC2 was also verified by *in vitro* reaction (Fig. 4). The RAD18-induced ubiquitylation of RFC2 *in vitro* and in RAD18-over-expressing cultured cells is similar to what we and others have observed for PCNA, a bona fide RAD18 substrate. These results are further indicative of a direct E3 ligase-substrate relationship between RAD18 and RFC2.

Our *in vitro* experiments clearly show an inhibitory effect of RPA on RFC2 mono-ubiquitylation (Fig. 4). The involvement of RPA in regulation of RFC2 ubiquitylation *in vivo* is also suggested by our experiments with the RFC2-D228A mutant (corresponding to a *S. cerevisiae* RPA interaction-deficient Rfc4 mutant). We have shown that RFC2-D228A undergoes DNA damage-independent ubiquitylation, which is reduced substantially in RAD18-deficient cells (Fig. 3C). Our *in vitro* assay for RAD6-RAD18-dependent RFC2 ubiquitylation does not completely recapitulate all aspects of RFC2 modification *in vivo*, and the role of RFC2 D228 in mediating RPA associations is not yet clear. However, our results strongly suggest a key regulatory role of RPA in RFC2 ubiquitylation. We propose that Rad18-dependent RFC2 ubiquitylation is repressed by RPA in undamaged cells, and that de-repression of RFC2 ubiquitylation occurs following MMS-induced DNA damage.

Our experiments also indicate that the RFC2-D228A mutant is subject to extensive poly-ubiquitylation. It is likely that poly-ubiquitylated RFC2 is generated by linkage of additional ubiquitin molecules to lysine residues that are first mono-ubiquitylated by RAD18. By analogy, following genotoxin treatments PCNA is mono-ubiquitylated by RAD6-RAD18 on lysine-164, and subsequently the mono-ubiquitylated PCNA is poly-ubiquitylated in a reaction mediated by MMS2-UBC13 and RAD5 (22,23,42,43). It will be interesting to determine whether RAD5 or alternative E3 ligases contribute to the RAD18-initiated poly-ubiquitylation of RFC2. Mono-ubiquitylated and poly-ubiquitylated species of PCNA promote different damage response pathways, error-prone and error-free post-replication repair (PRR), respectively. It will be interesting to determine whether the mono- and poly-ubiquitylated species of RFC2 similarly serve distinct effector functions. Several studies have demonstrated that a residual level of PCNA ubiquitylation is detectable in RAD18-deficient cells. Similarly, we have shown that RAD18-deficiency does not completely ablate RFC2 ubiquitylation. Clearly, further work is necessary to identify the E3 ligases involved in RAD18-independent ubiquitylation of PCNA and RFC2.

In order to obtain insight into the question of why the RFC2 D228A mutant is susceptible to ubiquitylation without DNA damage, we constructed tertiary structure models of human RFC2 (Fig. 3A) and RFC complex bound to

PCNA (Fig. 5) by homology modeling using the reported yeast structure (41) as the template. Each RFC subunit contains three structurally conserved domains (Domain I, II and III). Domains I and II comprise an ATPase module of the AAA+ family, which is connected by a flexible linker to another helical domain (Domain III). Our structural model revealed that D228 resides in the turn between helix14 and helix15 (Sensor 2 helix), which is located near the hinge region between Domain II and III (Fig. 5C). This implies that RFC2-D228 is not exposed to the outer surface, but instead is buried in the spiral structure. It is unlikely, therefore, that the D228 residue directly associates with RPA, as long as such a tight RFC-PCNA complex is maintained.

Whether the RFC complex remains around primed end following PCNA loading is controversial (11,30,44-46). However, the RFC complex may stay associated with PCNA in a structure different from the tight complex as shown in Fig. 5A, which allows RPA to associate with RFC2 around the D228 residue. Another possibility is that the D228A mutation causes a conformational change in the RFC complex structure, possibly altering interactions with RPA and affecting susceptibility to ubiquitylation.

It is notable that RFC2 is ubiquitylated in human cells following treatment with alkylating agents, but not in response to genotoxins that induce DSB, bulky adducts, ICL, or nucleotide depletion. Therefore, it appears that RFC2 mono-ubiquitylation is due to a specific alteration in DNA structure induced by alkylating agents or to a specific DNA repair intermediate. Identification of DNA structure(s) responsible for RFC2 ubiquitylation may provide insight into consequences of DNA damage due to particular genotoxins. Alkylating agents modify DNA by adding methyl or ethyl groups to a number of nucleophilic sites on the DNA bases (47). The predominant adduct in double strand DNA resulting from MMS or MNNG exposure is 7-methylguanine (*N*7-MeG) and 3-methyl adenine (*N*3-MeA). *N*3-MeA blocks replication, whereas *N*7-MeG does not block replication or miscode. Another deleterious adduct is ^{6}*O*-methyl guanine (^{6}*O*-MeG). ^{6}*O*-MeG is produced relatively lower level compared to *N*7-MeG and *N*3-MeA, but highly mutagenic and toxic, since ^{6}*O*-MeG-T mispairing not only results in G/C to A/T transition but also is recognized by mismatch repair (MMR) in process that is a potent signal of apoptosis (48). However, the human kidney cell line 293A cells, used in this study, are MMR-deficient, due to epigenetic silencing of the *hMLH1* gene by promoter hypermethylation (49). Therefore. ^{6}*O*-MeG is not the lesion responsible for RFC2 monoubiquitylation and instead *N*7-MeG and/or *N*3-MeA are the likely candidates. Treatment of 293A cells with an oxidative agent (H_2O_2) also induced RFC2 monoubiquitylation (Fig. 1E).

Base excision repair (BER) is the common pathway for repairing *N*7-MeG, *N*3-MeA and oxidative damage (47,50,51). BER is initiated with removal of altered bases by DNA-glycosylase. The resulting apurinic/apyrimidinic (AP) sites are nicked and repair is completed by re-synthesis and ligation. Therefore, for proficient BER, a proper balance of the individual steps involved in DNA repair is important. Imbalanced BER may result in deleterious intermediates, such as AP sites. Furthermore, methylation or oxidation of purines destabilizes the *N*-glycosyl bond, thus rendering the base more susceptible to hydrolysis to form an AP site. Therefore, AP sites are the lesions most likely to cause RFC2 monoubiquitylation, although precisely how RPA-RFC2 interaction is affected at AP sites is unclear.

Another possible role of RFC2 ubiquitylation is as the sensing signal for damage recognition. The RFC1-5 complex (containing RFC2) has several functions. During normal DNA replication RFC1-5 acts as clamp loader for PCNA, whereas in the DNA damage response RAD17-RFC2-5 loads the 9-1-1 complex. At present we do not know whether loading of PCNA, the 9-1-1 complex, or both is affected by RAD18-dependent RFC2 modification. Experiments to further address the significance of RFC2 modification and to identify relevant effectors of modified RFC are underway.

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