Monoubiquitination of Proliferating Cell Nuclear Antigen Induced by Stalled Replication Requires Uncoupling of DNA Polymerase and Mini-chromosome Maintenance Helicase Activities

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Proliferating cell nuclear antigen (PCNA) is a homotrimeric, ring-shaped protein complex that functions as a processivity factor for DNA polymerases. Following genotoxic stress, PCNA is modified at a conserved site by either a single ubiquitin moiety or a polyubiquitin chain. These modifications are required to coordinate DNA damage tolerance processes with ongoing replication. The molecular mechanisms responsible for inducing PCNA ubiquitination are not well understood. Using Xenopus egg extracts, we show that ultraviolet radiation and aphidicolin treatment induce the mono- and diubiquitination of PCNA. PCNA ubiquitination is replication-dependent and coincides with activation of the ataxia telangiectasia mutated and Rad3-related (ATR)-dependent DNA damage checkpoint pathway. However, loss of ATR signaling by depletion of the ATR-interacting protein (ATRIP) or Rad1, a component of the 911 checkpoint clamp, does not impair PCNA ubiquitination. Primed single-stranded DNA generated by uncoupling of mini-chromosome maintenance helicase and DNA polymerase activities has been shown previously to be necessary for ATR activation. Here we show that PCNA ubiquitination also requires uncoupling of helicase and polymerase activities. We further demonstrate that replicating single-stranded DNA, which mimics the structure produced upon uncoupling, is sufficient to induce PCNA monoubiquitination. Our results suggest that PCNA ubiquitination and ATR activation are two independent events that occur in response to a common single-stranded DNA intermediate generated by functional uncoupling of mini-chromosome maintenance (MCM) helicase and DNA polymerase activities.

Bulky lesions in the DNA cause arrest of replicative polymerases, halting cell cycle progression and activating DNA damage checkpoints. DNA damage tolerance mechanisms allow the cell to continue replication under these conditions and permit repair of the damage at a later time (1). Translesion synthesis (TLS)2 is one type of DNA damage tolerance mechanism that allows specialized, low fidelity DNA polymerases to bypass DNA damage in an error-prone fashion. A second mechanism of DNA damage tolerance involves error-free strategies of bypass that include post-replication recombinational repair and replication fork regression. How these DNA damage tolerance mechanisms are coordinated with replication at stalled forks is largely unknown.

One protein that plays a critical role in linking these processes is proliferating cell nuclear antigen (PCNA). PCNA is a homotrimeric ring-shaped protein complex that functions as a processivity factor necessary to maintain ongoing synthesis of DNA. PCNA has critical functions in DNA replication and DNA repair of the damage at a later time (1). Translesion synthesis (TLS)2 is one type of DNA damage tolerance mechanism that allows specialized, low fidelity DNA polymerases to bypass DNA damage in an error-prone fashion. A second mechanism of DNA damage tolerance involves error-free strategies of bypass that include post-replication recombinational repair and replication fork regression. How these DNA damage tolerance mechanisms are coordinated with replication at stalled forks is largely unknown.

One protein that plays a critical role in linking these processes is proliferating cell nuclear antigen (PCNA). PCNA is a homotrimeric ring-shaped protein complex that functions as a processivity factor necessary to maintain ongoing synthesis of DNA. PCNA has critical functions in DNA replication and DNA damage tolerance, and these functions depend on its interaction with other proteins. In addition, post-translational modification of PCNA has emerged as a critical step in regulating PCNA functions (1, 2).

In response to DNA damage or replication fork stalling, PCNA is modified at a conserved site, Lys-164, by either a single ubiquitin moiety or a polyubiquitin chain, linked via Lys-63 (3–7). These ubiquitination events promote DNA damage tolerance processes in either an error-prone or error-free manner. Monoubiquitination of PCNA activates TLS by facilitating the recruitment of error-prone TLS polymerases such as polymerase η to PCNA at the replication fork (5, 8, 9). The ubiquitin moiety on PCNA is recognized by ubiquitin-binding domains found in members of all Y family TLS polymerases (5, 10). In contrast, polyubiquitination of PCNA is thought to promote the error-free mechanisms of DNA damage tolerance (4, 6).

PCNA ubiquitination has been shown to occur following several types of genotoxic stress, including treatment with ultraviolet radiation (3–5, 7, 8), methyl methanesulfonate (3, 4), hydroxyurea (5), and aphidicolin (7). These DNA-damaging agents are also known activators of the ataxia-telangiectasia and Rad3-related (ATR)-dependent checkpoint pathway (11, 26).
In response to these agents, the ATR kinase, in complex with its cofactor ATR-interacting protein (ATRIP), phosphorylates and activates the effector kinase Chk1. Phosphorylation of numerous substrates by ATR and Chk1 leads to arrest of cell cycle progression and activation of pathways that stabilize stalled replication forks (12, 13). Activation of the ATR-mediated checkpoint pathway by UV radiation, methyl methanesulfonate, and aphidicolin in Xenopus egg extracts is a replication-dependent process (14, 15) that requires functional uncoupling of the MCM helicase and DNA polymerase activities (16). Upon encounter of a bulky lesion or direct inhibition by aphidicolin, the polymerase is stalled, whereas the helicase continues to unwind DNA (16–18). This leads to the accumulation of single-stranded DNA (ssDNA) stabilized by the ssDNA-binding protein, replication protein A (RPA) (17, 18), a component of the signal required for activation of the ATR checkpoint response (19).

The similarity in signals leading to PCNA ubiquitination and ATR activation as well as the role of ATR in maintaining replication fork stability raise the possibility that the ATR pathway might regulate the ubiquitination of PCNA. Here we used the Xenopus egg extract system to study PCNA ubiquitination. Addition of sperm chromatin to low speed supernatants (LSS) of Xenopus egg cytoplasm results in the assembly of pre-replication (pre-RC) complexes and nuclear envelope formation. Initiation of DNA replication then occurs, and DNA is replicated in a semi-conservative fashion (20). Using this cell-free system, we find that UV and aphidicolin treatment induce PCNA mono- and polyubiquitination in Xenopus egg extracts, an event consistent with studies in yeast and mammalian cells. PCNA ubiquitination coincides with activation of the ATR-mediated checkpoint response; however, loss of ATR activity does not impair PCNA monoubiquitination. Instead, we show that PCNA ubiquitination and ATR activation are two parallel events that occur in response to a common ssDNA intermediate generated by functional uncoupling of MCM helicase and DNA polymerase activities.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Recombinant Proteins**

Antibodies used for immunodepletion and immunoblotting of Xenopus laevis Rad1 (14) and X. laevis ATRIP (16) were produced as described. Antibodies against PCNA (PC-10 sc-56), Chk1 (G-4 sc-8408), and GST (B-14 sc-138) were purchased from Santa Cruz Biotechnology. The phospho-Ser-344 Chk1 (G-4 sc-8408), and GST (B-14 sc-138) were purchased from Cell Signaling Technology. The phospho-Ser-344 Chk1 antibody was raised by Zymed Laboratories Inc. X. laevis Orc2 and RPA antibodies were provided by P. Jackson (Stanford University) and J. Walter (Harvard University). Purified Cdc45 IgG and recombinant His6-Cdc45 were generously supplied by J. Walter (Harvard University). Recombinant GST-tagged p27KIP was expressed and purified as described (17). Recombinant His6-geminin was preincubated with egg extracts at a final concentration of 750 nM as described previously (14).

Full-length X. laevis PCNA was cloned from IMAGE: 5049027 using the following primers: 5′-CGGGATCCATGT-TTGAGGCTCGCTTG-3′ and 5′-CCGCTCGAGGATTTGAC-3′. The K164R PCNA mutant was constructed using site-directed mutagenesis with the following primers: 5′-CAGTAGATTTCTTGTGCCCCGATGGG-GTAAATTC-3′ and 5′-GAACCTTTTACCCCATCCCGGCC-AACAGAAAATCTACTG-3′. Both constructs were confirmed by sequencing. PCR products (wild-type and K164R mutant) were digested with BamHI and Xhol and ligated into pET28 (Novagen). The His fusion proteins were expressed in Escherichia coli BL21(DE3) at 37 °C and purified by affinity chromatography using Ni2+-agarose (Sigma).

A pET3a construct containing ubiquitin from Arabidopsis thaliana was kindly provided by P. Jackson (Stanford University). BamHI and Xhol sites were added in-frame with ubiquitin by PCR using the following primers: 5′-CGGGATCCACCAT-GCAGATATTCGTAAGAC-3′ and 5′-CCGCTCGAGTTA-ACCACAGGACCGGAG-3′. The resulting PCR product was cut and ligated into pGEX4T3 (Amersham Biosciences) at the BamHI and Xhol sites. GST-tagged ubiquitin was expressed in E. coli BL21(DE3) at 37 °C and purified using GST-agarose (Sigma). Recombinant His6-ubiquitin was kindly provided by G. Fang (Stanford University).

**Xenopus Egg Extract Preparation and DNA-damaging Agents**

Xenopus egg extracts (LSS, HSS, and NPE) were prepared as described (14, 16, 21). Xenopus sperm chromatin was UV-irradiated at 1000 J/m2 using a Stratalinker (Stratagene). Chromatin was used at 2,500 nuclei/μl unless otherwise indicated. Aphidicolin (Sigma) was dissolved in Me2SO at a stock concentration of 30 mM and stored at −20 °C in single-use aliquots. Caffeine was dissolved in 10 mM PIPES-KOH, pH 7.7, and used at a final concentration of 4 mM as described previously (14). M13 ssDNA was phenol/chloroform-extracted and ethanol-precipitated. The circular DNA form was then gel-purified.

**Immunodepletions, Nuclear Isolation, Chromatin Binding, and Replication Assays**

Immunodepletions of ATRIP and Rad1 were performed as three consecutive 30-min incubations at 4 °C using Affigel protein-A beads (Bio-Rad) as described (16). Nuclei and chromatin were isolated from LSS (14) and HSS/NPE (17) using a sucrose cushion as published. Replication assays were performed as described previously (14).

**Pulldown Assays**

GST Pulldown from LSS—Nuclei were resuspended in 100 μl of PBS containing 0.5% Triton X-100. The nuclear lysate was incubated with 10–15 μl of pre-equilibrated GST-agarose and rotated at 4 °C for 30 min. The beads were washed six times with 0.5% Triton X-100/PBS followed by addition of SDS sample buffer.

GST Pulldown from HSS—Samples were diluted with 50 μl of PBS and added to 15 μl of pre-equilibrated GST-agarose. The beads were processed as described above.

Ni2+ Pulldown from LSS—Nuclei were isolated at 110 min, resuspended in 100 μl of Buffer A (30 mM Na2HPO4, pH 8, 300 mM NaCl, 0.5% Triton X-100), and incubated with 20 μl of pre-equilibrated Ni2+-agarose (Sigma). The beads were processed as described above and washed with Buffer A containing 10 mM imidazole.
**RESULTS**

**Monoubiquitination of PCNA Is Induced by Genotoxic Stress in a Replication-dependent Manner**—Treatment of chromatin with UV or aphidicolin induces activation of the ATR-dependent checkpoint in *Xenopus* egg extracts, as monitored by phosphorylation of Chk1 at Ser-344 (Fig. 1, A, C, and F) (22, 23). To determine the effect of these agents on PCNA, we analyzed the chromatin-bound form of PCNA after UV and aphidicolin treatment. Both agents led to the accumulation of a form of PCNA ~8 kDa larger than PCNA itself (Fig. 1A), an increase consistent with the attachment of a single ubiquitin moiety.

To verify that PCNA is monoubiquitinated following genotoxic stress, we added purified GST-ubiquitin to LSS prior to addition of UV-damaged chromatin or aphidicolin and isolated glutathione-bound proteins. Following either treatment, we obtained a protein with a molecular mass corresponding to mono-GST-ubiquitinated PCNA that was recognized by PCNA and GST antibodies (Fig. 1B). We observed similar results using His₆-tagged ubiquitin (supplemental Fig. 1A). These observations confirm that PCNA is monoubiquitinated in *Xenopus* egg extracts following several types of genotoxic stress.

Mutation of a conserved lysine (Lys-164) to arginine has been shown to abrogate PCNA ubiquitination in both yeast (3, 4, 24) and mammalian cells (5). To determine whether the DNA damage-induced monoubiquitination of PCNA also occurs at Lys-164 in *Xenopus* egg extracts, we added either wild-type His₆-tagged *Xenopus* PCNA (H₆xPCNA) or the K164R mutant of H₆xPCNA to LSS and then used nickel resin to isolate these proteins from nuclei following UV or aphidicolin treatment. As expected, wild-type H₆xPCNA was monoubiquitinated in the presence of aphidicolin or UV-treated chromatin, but the Lys-164 mutant was not (Fig. 1C).

To better characterize this post-translational modification, we analyzed PCNA monoubiquitination at varying times in the presence and absence of UV damage. Monoubiquitination was first observed 15–30 min after addition of UV-damaged chromatin to LSS and was coincident with the onset of DNA replication (Fig. 1D). In the absence of DNA damage, only minor amounts of monoubiquitination were noted over the course of the entire experiment. We also analyzed PCNA monoubiquitination over a range of aphidicolin concentrations that affect the extent of replication inhibition (16) and the accumulation of RPA-coated single-stranded DNA (Fig. 1E). We found PCNA to be monoubiquitinated at all concentrations tested. In addition, we observed another form of PCNA with a molecular mass consistent with diubiquitination at higher concentrations of aphidicolin. To confirm that this additional form of PCNA is diubiquitinated PCNA, we isolated PCNA from extracts using p21 peptide resin (25) and probed for ubiquitin at two concentrations of aphidicolin. Both higher mobility forms of PCNA were recognized by an anti-ubiquitin antibody, and longer ubiquitin chains were also detected (supplemental Fig. 1B). Taken together, our data indicate that although a small amount of PCNA monoubiquitination may occur during unperturbed replication, both mono- and diubiquitination are strongly induced by DNA damage. These findings are consistent with observations in yeast (3, 4, 24) and mammalian cells (5, 8).

**PCNA Monoubiquitination Occurs Independently of the ATR-mediated Checkpoint Pathway**—The similarity in signals leading to PCNA ubiquitination and ATR activation (Fig. 1A, D), as well as the roles of the ATR pathway in maintaining replication fork stability and DNA damage-induced mutagenesis (12, 26–29), raised the possibility that ATR activation and PCNA ubiquitination might be linked. Thus, we tested whether UV and aphidicolin-induced PCNA monoubiquitination, like ATR activation with these agents (14, 15), is an S-phase specific event. Addition of geminin, which inhibits replication by preventing assembly of the pre-RC (20), abrogated aphidicolin and UV-induced PCNA monoubiquitination and, as a control, Chk1 phosphorylation (Fig. 1F). This observation indicates that PCNA monoubiquitination in response to UV and aphidicolin is a replication-dependent process in *Xenopus* egg extracts.

We then assessed the effect of blocking the ATR-mediated checkpoint on PCNA monoubiquitination. First, we used caffeine, an inhibitor of both the ATM- and ATR-mediated checkpoint responses (30). As shown in Fig. 2A, caffeine abrogated aphidicolin-induced Chk1 phosphorylation (total lysate, compare lanes 3 and 4), whereas monoubiquitination of PCNA increased (chromatin fraction, compare lanes 3 and 4). These data suggest that PCNA monoubiquitination is not dependent on ATM or ATR kinase activity. To test more directly the dependence of PCNA monoubiquitination on ATR, we immunodepleted ATRIP, an ATR-binding protein required for ATR signaling, from LSS. Consistent with previous reports (16, 31), depletion of ATRIP prevented ATR-dependent Chk1 phosphorylation following UV or aphidicolin treatment (Fig. 2B, total lysate). However, as seen with caffeine, PCNA monoubiquitination was not blocked and modestly increased upon depletion of ATRIP (Fig. 2B, chromatin fraction).

The Rad9-Hus1-Rad1 (911) checkpoint clamp is another essential component of the ATR-mediated checkpoint pathway that has been suggested to have a role in translesion synthesis (26, 28, 32, 33). Because monoubiquitinated PCNA is also involved in translesion synthesis, we asked whether the 911 complex is necessary for PCNA monoubiquitination. Immunodepletion of Rad1 abrogated ATR-dependent Chk1 phosphorylation, as reported previously (34) (Fig. 2C, total lysate, compare lanes 3 and 4). However, aphidicolin-inducible PCNA monoubiquitination was not inhibited by depletion of the 911 checkpoint clamp (Fig. 2C, chromatin fraction, compare lanes 3 and 4), indicating that PCNA monoubiquitination does not require 911 function. The increase in PCNA monoubiquitination observed upon treatment with caffeine or depletion of ATRIP or Rad1 is likely because of the inability to prevent late origin firing in the absence of the ATR-dependent checkpoint and the loading or persistence of PCNA at replication forks.
FIGURE 1. Genotoxic stress induces replication-dependent monoubiquitination of PCNA in extracts of *X. laevis*. A, Xenopus sperm chromatin (2,500 nuclei/µl) was undamaged (mock), UV-irradiated (1000 J/m²), or treated with aphidicolin (Aph, 15 µM) and added to LSS of Xenopus egg extracts. At 90 min, an aliquot of total lysate was taken to assay for Chk1 phosphorylation using a phospho-specific Chk1 antibody (Chk1 P-S344), and then chromatin was isolated by spinning through a sucrose cushion. Proteins were separated by SDS-PAGE and analyzed by Western blot using the indicated antibodies. ORC2 levels were monitored as a loading control for the chromatin fraction. Ubiquitinated PCNA (PCNA-Ub) is indicated. B, mock or UV-treated (1000 J/m²) chromatin was added to LSS containing GST-tagged ubiquitin (GST-Ub) at the indicated concentrations. Aphidicolin (15 µM) was added to chromatin prior to addition of LSS. An aliquot of total lysate was removed at 90 min and analyzed as in A. Nuclei were isolated from the remaining sample, and nuclear lysates were subjected to a GST pull-down assay followed by Western blot with the indicated antibodies. GST-ubiquitinated PCNA (PCNA-GSTUb) is indicated. C, LSS were supplemented with PBS (−), wild-type (wt) His-tagged PCNA (HisPCNA), or the K164R mutant HisxPCNA (K164R). UV-damaged or aphidicolin-treated (15 µM) sperm chromatin was then added. Nuclei were isolated, and nuclear lysates were subjected to a HisxPCNA pull-down assay and bound proteins analyzed by Western blot. His-ubiquitinated PCNA (HisPCNA-Ub) is indicated. D, undamaged (mock) or UV-irradiated (1000 J/m²) Xenopus sperm chromatin was added to LSS. At t = 0 a sample was removed and incubated with trace amounts of (α-32P)dATP to monitor for replication, as shown in the graph. The remaining sample was analyzed at the indicated time points as described in A. E, sperm chromatin was mixed with the indicated concentrations of aphidicolin followed by addition of LSS. Chromatin-bound proteins were processed and analyzed as described in A after 90 min. Mono- (PCNA-Ub) and diubiquitinated PCNA (PCNA-Ub2) are indicated. F, LSS were incubated with the replication inhibitor geminin or an equal volume of buffer for 10 min prior to addition of undamaged (mock) or UV-irradiated (1000 J/m²) sperm chromatin. Aphidicolin (150 µM) was added to mock-treated chromatin preceding addition to LSS. Samples were isolated and analyzed after 90 min as described in A.
PCNA Monoubiquitination Requires Uncoupling

Taken together, these results indicate that monoubiquitination of PCNA induced by UV or aphidicolin can occur in the absence of ATR-ATRIP and 911 function. Moreover, the persistence of PCNA monoubiquitination in the absence of Chk1 phosphorylation suggests that PCNA monoubiquitination is independent of Chk1 phosphorylation.

Monoubiquitination of PCNA Requires Cdc45-mediated Uncoupling of DNA Polymerase and MCM Helicase Activities—Rad18, the ubiquitin-protein isopeptide ligase required for PCNA monoubiquitination, binds ssDNA in vitro (1, 9, 36) and co-localizes with PCNA and polymerase η, an error-prone DNA polymerase involved in translesion synthesis, following UV damage in mammalian cells (8). This raises the possibility that PCNA ubiquitination is induced by the same signal that activates the ATR pathway—primed ssDNA generated at stalled forks through functional dissociation of the MCM2–7 helicase and DNA polymerase activities (16, 17). To test this possibility, we inhibited uncoupling of helicase and polymerase activities by neutralization of Cdc45, an essential MCM helicase cofactor, using a protocol published previously (17).

For this experiment, chromatin was incubated first in HSS of Xenopus egg extracts to allow for pre-RC formation. After 30 min, NPE was added to initiate replication and elongation by providing high levels of cdK2 and cdK7 kinase activities (20). The sequential addition of HSS and NPE to chromatin allows for analysis of replication in the absence of nuclear formation (21). Following initiation of replication, GST-p27KIP was added to prevent any subsequent origin firing. Chromatin was isolated before elongation was completed and then mock-treated or incubated with neutralizing antibodies raised against Cdc45. To allow resumption of replication, the treated chromatin was then released into fresh NPE in the presence or absence of aphidicolin, and 75 min later, chromatin was reisolated and PCNA ubiquitination was assessed.

As shown in Fig. 3, addition of 30 μM aphidicolin led to a significant increase in PCNA monoubiquitination and the appearance of a small amount of diubiquitinated PCNA (PCNA-Ub2), as compared with mock treatment (lanes 1 and 2). Neutralization of Cdc45 activity by addition of purified Cdc45 antibodies largely abolished PCNA ubiquitination in the presence of aphidicolin (Fig. 3, lane 3). As a control, preincubation of Cdc45-neutralizing antibodies with recombinant His6-Cdc45 restored aphidicolin-inducible ubiquitination of PCNA (Fig. 3, lane 4), confirming that the observed loss of PCNA ubiquitination is because of neutralization of Cdc45. Consistent with previous results (16), aphidicolin-inducible Chk1 phosphorylation was also blocked by addition of Cdc45-neutralizing antibodies. These results indicate that Cdc45-mediated uncoupling of helicase and polymerase activities is necessary for ubiquitination of PCNA.

Replicating Single-stranded DNA Induces Monoubiquitination of PCNA—Upon addition to high speed supernatants (HSS) of Xenopus egg extracts, single-stranded M13 (ssM13) DNA is primed and replicated in a PCNA- and polymerase α-dependent manner (25, 37). Thus, replication of the ssM13 DNA generates an intermediate that mimics the ssDNA formed ahead of a stalled polymerase during helicase-polymerase uncoupling. To further test the idea that primed ssDNA is the signal that activates PCNA ubiquitination, we asked if this replicating ssDNA was sufficient to induce PCNA ubiquitination in Xenopus egg extracts. As shown in Fig. 4A, addition of ssM13 DNA to HSS containing GST-tagged ubiquitin triggered the formation of mono-GST-ubiquitinated PCNA (Fig. 4A, lane 2).

FIGURE 2. Monoubiquitination of PCNA does not require ATR or Rad1. A, caffeine (4 mM) or buffer was added to LSS as indicated followed by addition of Xenopus sperm chromatin premixed with aphidicolin (Aph) (150 μM) or buffer. B, LSS were immunodepleted using either rabbit IgG (ΔIgG) or ATRIP antibodies (ΔATRIP) followed by addition of either undamaged (mock) or UV-irradiated (1000 J/m2) chromatin. Aphidicolin was used at 15 μM. C, LSS were immunodepleted using either rabbit IgG (ΔIgG) or Rad1 antibodies (ΔRad1) followed by addition of chromatin premixed with aphidicolin (150 μM) or buffer. In all cases, samples were isolated, and proteins were analyzed as described in Fig. 1A.

FIGURE 3. Cdc45-mediated uncoupling of the MCM helicase and DNA polymerase activities is necessary for DNA damage-inducible PCNA ubiquitination. Xenopus sperm chromatin (10,000 nuclei/μl) was incubated in 5 μl of HSS for 30 min, and then 5 μl of NPE was added. After 15 min, GST-p27KIP (2 μM) was added to all samples, and 10 min later chromatin was isolated and treated with buffer (lanes 1 and 2), Cdc45 IgG (lane 3), or Cdc45 IgG premixed with recombinant His6-Cdc45 (rCdc45, lane 4). Replication was reinitiated by addition of 5 μl of NPE containing 30 μM aphidicolin (lanes 2–4) or buffer (lane 1). After an additional 75 min, an aliquot of total lysate was taken, and then chromatin was isolated and proteins were analyzed as described in Fig. 1A.
PCNA Monoubiquitination Requires Uncoupling

To test the requirement for replication of the ssDNA in PCNA ubiquitination, we also added a dose of aphidicolin sufficient to block all detectable DNA replication (Fig. 4B) (16). Treatment with aphidicolin led to a substantial decrease in mono-GST-ubiquitinated PCNA (Fig. 4A, compare lanes 2 and 3). These results indicate that replicating ssDNA is sufficient to induce PCNA ubiquitination.

**DISCUSSION**

Our findings indicate that mono- and diubiquitination of PCNA at lysine 164 are strongly induced by UV or aphidicolin treatment in *Xenopus* egg extracts. We also show that Cdc45-dependent uncoupling of helicase and polymerase activities is needed for aphidicolin-induced ubiquitination of PCNA, but the ATR-ATRIP and 911 complexes are not. Moreover, replicating ssDNA, which mimics the DNA intermediate formed upon Cdc45-dependent uncoupling, can induce this post-translational modification. These data support a model in which PCNA ubiquitination is initiated by ssDNA that accumulates at stalled replication forks.

Recently it was shown that primed ssDNA can induce PCNA ubiquitination *in vitro* using purified proteins. Although elegant studies, the role of ssDNA in these experiments is difficult to discern because ubiquitination only occurred when PCNA was loaded onto the DNA (38, 39). It is likely that the requirement for replication of ssDNA in our experiments also reflects the need for PCNA to load onto DNA. Helicase-polymerase uncoupling leads to the accumulation of RPA-coated ssDNA and occurs following treatment with agents that inhibit DNA polymerases (16–18). Thus, our finding that Cdc45-mediated helicase-polymerase uncoupling is required for PCNA ubiquitination provides the first direct evidence that the additional accumulation of ssDNA is necessary for this event. The ability of replicating ssDNA to induce PCNA ubiquitination in cell-free egg extracts is also consistent with this idea and further demonstrates that neither a lesion nor the stalled fork itself is required for this response.

We recently reported that activation of ATR following UV and aphidicolin also requires uncoupling of MCM helicase and polymerase activities (16), and we have found that replicating ssDNA can induce ATR activation. In addition, PCNA ubiquitination and ATR-dependent Chk1 phosphorylation occur in response to similar types of stress with similar kinetics (Fig. 4A and D). Together, these observations suggest that PCNA ubiquitination and ATR-mediated checkpoint activation are induced by a common signal. Importantly, however, these events appear to be independent of each other. Loss of the checkpoint mediators ATRIP or Rad1 does not prevent PCNA ubiquitination (Fig. 2, B–C) nor does loss of PCNA prevent ATR-dependent phosphorylation of Chk1 (24) (data not shown). Although PCNA ubiquitination is not dependent on the homolog of ATR, Rad3, in *Schizosaccharomyces pombe* (24), results in mammalian cells suggest a partial dependence on ATR, Chk1, and RPA (40). These findings could reflect a difference between yeast, *Xenopus*, and mammalian cells, although it is also conceivable that in mammalian cells the loss of ATR, which leads to genomic instability and cell death (41–43), perturbs the cell cycle and DNA replication over the course of this experiment. This could cause an indirect effect on the ubiquitination of PCNA, a replication-dependent event. It is also important to note that our results do not preclude a role for ATR or the 911 complex in regulation of TLS or in error-free repair, as has been suggested previously (26–28, 33). Although these proteins do not appear to be required for PCNA ubiquitination, they may be needed for other aspects of DNA damage tolerance or DNA repair.

We hypothesize that primed ssDNA generated upon Cdc45-mediated uncoupling of helicase and polymerase activities acts as a critical signaling intermediate for multiple aspects of the DNA damage response, including those leading to checkpoint activation and DNA damage tolerance. It appears likely that this structure mediates the assembly of protein complexes involved in these processes, with ssDNA recruiting ATR-ATRIP (44, 45) and possibly the Rad6-Rad18 and Rad5-Mms2-Ubc13 complexes, the latter two of which mediate mono- and diubiquitination of PCNA (1, 9). Primers then serve to recruit the 911 complex (16, 46) to initiate activation of the checkpoint.

3 C. MacDougall, T. Byun, and K. A. Cimprich, unpublished data.
and the bound PCNA is ubiquitinated to facilitate bypass of lesions at the stalled fork (Fig. 5). Recent studies have shown that the enzyme that deubiquitates PCNA is regulated following DNA damage by autolysis (47). Interestingly, this regulation is also independent of the ATR pathway. Thus, it is possible that ssDNA at the replication fork regulates ubiquitination and/or deubiquitination of PCNA.

Although our results are consistent with findings in yeast and mammalian cells showing that DNA damage induces monom- and polyubiquitination of PCNA, a recent paper reported that sumoylation and monoubiquitination of PCNA occur during the replication of undamaged DNA in Xenopus egg extracts (7). These authors also report that monoubiquitination is unaffected by DNA damage, which under their conditions induces diubiquitination. We have also observed this result, but only when high concentrations of sperm chromatin were added to extracts (e.g. 25,000 sperm chromatin/μL) (supplemental Fig. 2). These concentrations of sperm chromatin lead to poor formation of nuclei in egg extracts, and replication is also inefficient under these conditions, because of limiting amounts of proteins required for these processes (48–50). Similar concentrations of sperm chromatin (2,000–2,500/μL) were used in both studies; thus the reasons for the discrepancy in our results are still not clear. Based on the effect of adding high concentrations of sperm chromatin, one possibility is that differences in the quality of nuclei formation are responsible. However, it is also possible that different basal levels of DNA damage are incurred in the sperm chromatin during some experimental manipulation or that some other difference in the techniques used is responsible.

Finally, it is attractive to speculate that the amount of ssDNA generated at stalled replication forks could regulate the biological outcome of the DNA damage response. On this point, we note that the type of PCNA ubiquitination that occurs changes with the dose of aphidicolin and is correlated with the accumulation of RPA on chromatin (Fig. 1E). Monoubiquitination is induced at lower doses of aphidicolin where diubiquitination is undetectable, and at higher doses, diubiquitination is more prominent. We have shown previously that the aphidicolin dose modulates the extent and duration of DNA unwinding (16). Thus, these observations suggest that the persistence of replication fork stalling and ssDNA accumulation could affect the mechanism by which DNA damage is tolerated, with error-free pathways favored over the potentially error-prone TLS when damage is greater or more persistent.

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PCNA Monoubiquitination Requires Uncoupling

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