CTF18 interacts with replication protein A in response to replication stress

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Abstract. Replication stress response is a protective mechanism against defects in chromosome replication for maintaining genome integrity in eukaryotic cells. An alternative clamp loader complex termed chromosome transmission fidelity protein 18 and replication factor C (CTF18-RFC) has been shown to act as a positive regulator of two types of replication stress response: S-phase checkpoint signaling and translesion DNA synthesis. However, it remains largely unknown how CTF18-RFC responds to replication stress and is recruited to stalled replication forks. The present study demonstrated that endogenous CTF18 forms a physical complex with a single-stranded DNA-binding protein replication protein A (RPA) in mammalian cells. Using an *in situ* proximity ligation assay (PLA), it was demonstrated that the interaction between CTF18 and RPA occurs in chromatin when replication stress is elicited by treatment with hydroxyurea during S phase. Similar results were obtained after exposure to ultraviolet irradiation, which triggers translesion DNA synthesis. Furthermore, the PLA demonstrated that the kinetics of the interaction between CTF18 and RPA was positively correlated with that of checkpoint kinase 1 phosphorylation, which is an indicator of activation of the ATM and Rad3-related pathway. These findings provide novel insights into the molecular mechanism underlying the participation of CTF18-RFC in the regulation of replication stress response.

Introduction

Chromosome replication is a risky process for maintaining genome integrity, as unrepaired DNA lesions at S phase interfere with the progress of replication forks and thereby result in excessive formation of single-strand DNA (ssDNA) that could be a major cause of deleterious lesions, such as DNA double-strand breaks. To preserve genome integrity during chromosome replication, eukaryotic cells have acquired several adaptive responses to DNA damage (1,2). One of the most studied pathways is the S-phase checkpoint response, which is evoked by an exposed ssDNA at stalled replication forks, attributed to a deficiency in DNA synthesis. The checkpoint kinase ATM and Rad3-related (ATR) is recruited on ssDNA where it is coated with ssDNA-binding protein, replication protein A (RPA). It then causes the phosphorylation and activation of downstream checkpoint kinase 1 (Chk1), which in turn stabilizes replication forks for genome integrity (3-5). Another is a damage tolerance mechanism termed translesion DNA synthesis (TLS), the major process with which cells replicate past the unrepaired DNA lesion during S phase (6). When the normal replication machinery is blocked at ultraviolet (UV)-induced cyclobutane pyrimidine dimers (CPDs), the Y-family DNA polymerase Polh replaces the stalled replicative DNA polymerase. This is dependent upon monoubiquitination of the ring-shaped clamp protein, proliferating cell nuclear antigen (PCNA), by the E3 ubiquitin ligase RAD18. Monoubiquitinated PCNA has an increased affinity for Polh, thus aiding the recruitment of Polh to stalled replication forks and allowing accurate replicative bypass of CPDs by incorporating correct bases on the opposite strand (7,8). Consequently, TLS overcomes UV-induced replication blocks, thereby preventing sustained activation of the S-phase checkpoint in response to excessive formation of ssDNA (9).

Accumulating evidence has shown that the S-phase checkpoint and TLS are activated by conserved clamp loader complex termed chromosome transmission fidelity protein 18 and replication factor C (CTF18-RFC) (10-13). CTF18-RFC is one of four 'heteropentameric RFC complexes' each of which contains a common small subunit comprising RFC2-4 together with a unique larger subunit, including either RFC1, Elg1, RAD17 or CTF18. RFC1-RFC is important in normal DNA replication as it loads the homotrimeric PCNA clamp around the junction of primers with template DNA at replication forks (14). Elg1-RFC is involved in the maintenance of genome stability (15,16), while RAD17-RFC contributes to the activation of the DNA damage checkpoint by loading the heterotrimeric 9-1-1 checkpoint clamp at sites of damaged DNA (17). In addition, although CTF18-RFC was originally

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reported to be important in establishing sister chromatid cohesion (18), recent studies with budding yeast have shown that CTF18-RFC mediates activation of the S-phase checkpoint depending on the association with DNA polymerase ε (10). By contrast, a biochemical study with an *in vitro* reconstitution system has demonstrated that CTF18-RFC binds to and stimulates the DNA synthetic activity of DNA polymerase η (11). However, the molecular mechanisms underlying these alternative functions of CTF18-RFC remain largely unknown.

In the present study, it was demonstrated that RPA is a novel binding partner of CTF18 in mammalian cells. Among the heterotrimeric subunits, RPA1 and RPA2 are detected as a complex with CTF18. Notably, the DuoLink *in situ* proximity ligation assay (PLA) demonstrated the nuclear interaction between CTF18 and RPA in response to replication stresses induced by hydroxyurea (HU) treatment and UV irradiation. Furthermore, the kinetics of CTF18-RPA interaction were positively correlated with the sustained activation of the ATR-Chk1 pathway after UV irradiation. The present findings provide insight into the mechanism underlying the functional role of CTF18 in replication stress responses.

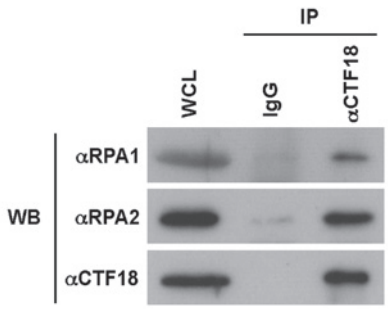

Figure 1. CTF18 forms a complex with RPA in HEK293 cells. Endogenous CTF18 interacts with RPA1 and RPA2. Whole-cell lysates from HEK293 cells were immunoprecipitated with normal IgG or anti-CTF18 antibody, followed by western blot analysis with antibodies as indicated. CTF18, chromosome transmission fidelity protein 18; RPA, replication protein A; IP, immunoprecipitation.

Materials and methods

Cell culture. HEK293 cells (RIKEN BioResource Center, Tsukuba, Japan) were kept at 37˚C in a humidified 5% CO_2 atmosphere and cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 100 units penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

Antibodies. The following antibodies were used in the present study: Rabbit polyclonal anti-CTF18 (cat no. A301-883A; 1:1,000 for western blot analysis; 1:100 for PLA; Bethyl Laboratories, Inc., Montgomery, TX, USA); rabbit monoclonal anti-RPA1 (cat. no. ab79398; 1:3,000 for western blot analysis) and mouse monoclonal anti-RPA2 (cat. no. ab2175; 1:2,000 for western blot analysis; 1:300 for PLA) (Abcam, Cambridge, MA, USA); mouse monoclonal anti-α tubulin (cat. no. B-5-1-2; 1:3,000 for western blot analysis; Sigma-Aldrich); mouse monoclonal anti-Chk1 (cat. no. K0086-3; 1:2,000 for western blot analysis; Medical & Biological Laboratories, Nagoya, Japan); and rabbit polyclonal anti-phospho-Chk1 at Ser345 (cat. no. 2341 1:1,000 for western blot analysis; Cell Signaling Technology, Danvers, MA, USA). Secondary antibodies were polyclonal HRP-conjugated sheep anti-mouse (cat. no. NA931V; 1:30,000) and donkey anti-rabbit (cat. no. NA934V; 1:30,000) obtained from GE Healthcare Life Sciences (Little Chalfont, UK).

Cell synchronization. Cell cycle synchronization was performed by the double thymidine block method as reported previously (19). Briefly, exponentially growing HEK293 cells were treated with 2 mM thymidine (Nacalai Tesque, Inc.) for 16 h, thymidine-free media for 10 h, and 2 mM thymidine for 18 h to arrest the cell cycle at the G1/S boundary. Then, cells were released by changing the medium and analyzed at various time intervals. Cell synchronization was conducted prior to the *in situ* proximity ligation assay and western blotting to detect phosphorylation of Chk1, however, the co-immunoprecipitation assay was performed with unsynchronized cells.

Co-immunoprecipitation assay and western blotting. Co-immunoprecipitation was performed as previously described (20). Briefly, whole-cell lysates were obtained from HEK293 cells using a lysis buffer [containing 20 mM Hepes (pH 7.9), 150 mM NaCl, 0.1% Triton X-100 ((Nacalai Tesque, Inc.) and protease inhibitor cocktail (Nacalai Tesque, Inc.)] were immunoprecipitated with normal IgG or anti-CTF18 antibody, and then separated by 10% SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA) and blocked for 1 h with 0.3% skim milk at room temperature. The membrane was incubated overnight at 4˚C with antibodies against RPA1, RPA2 and CTF18. Following washing with Tris-buffered saline with Tween 20 (TBST), the membrane was probed with the secondary antibodies for 1 h at room temperature and then washed again with TSBT. The membranes were visualized using the enhanced chemiluminescence system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and exposed to X-ray film (Fujifilm Corporation, Tokyo, Japan).

In situ proximity ligation assay (PLA). PLA was performed according to the manufacturer's instructions (Sigma-Aldrich). Briefly, synchronized HEK293 cells at S phase were treated with HU (0, 2 or 5 mM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) or irradiated with UV light (0, 20 or 100 J/m^2), after 2 h, cells were extracted with CSK buffer (10 mM PIPES-NaOH, pH 6.8; 300 mM sucrose and 100 mM NaCl) containing 0.5% Triton X-100 for 5 min for detection of chromatin bound proteins (21). After washing with CSK buffer without Triton X-100, cells were fixed with 3.7% formalin (Wako Pure Chemical Industries, Ltd.) for 20 min, followed by permeabilization with ice-cold methanol for 10 min. After blocking with Duolink Blocking solution (Sigma-Aldrich), cells were probed with mouse monoclonal anti-RPA2 and rabbit polyclonal anti-CTF18 antibodies, and then mouse or rabbit PLA probes were added. Hybridization of the oligonu-

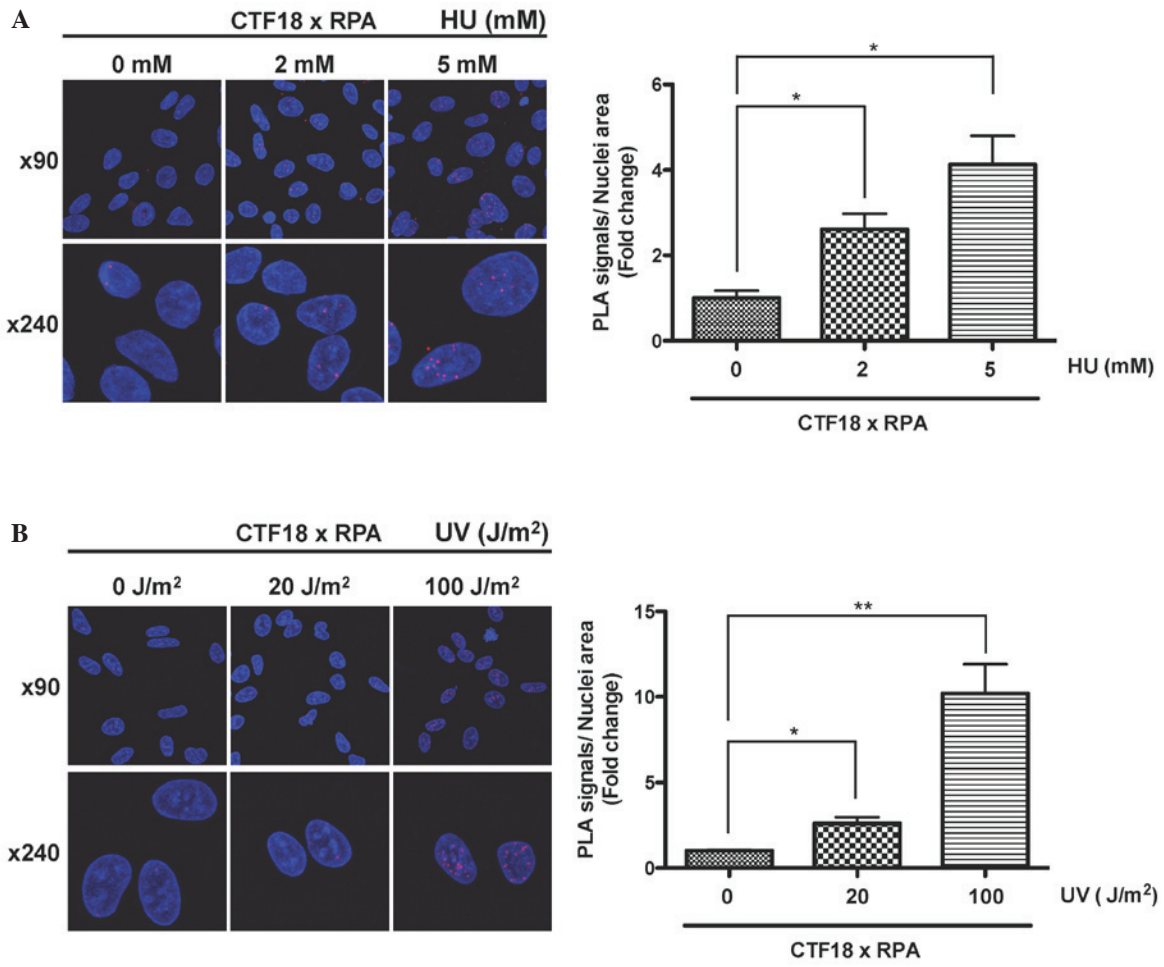

Figure 2. CTF18 binds to RPA in response to replication stresses. (A) The interaction between CTF18 and RPA occurs after treatment with HU. HEK293 cells were synchronized to S phase by double thymidine block and then treated with the indicated concentration of HU. After 2 h, cells were treated with CSK/Triton-X buffer followed by fixation, and then an *in situ* PLA was performed with anti-RPA2 and anti-CTF18 antibodies. The red fluorescent foci indicate the proximity of the two proteins (magnification, x90 or x240). Duolink Image Tool was used to quantify PLA signals (n=3, x90 magnification). The vertical axis shows the total nuclear PLA signals divided by nuclei area and normalized to non-treatment group, and the horizontal axis indicates the concentration of HU. Error bars indicate the standard error of the mean of three different fields. (B) The CTF18-RPA interaction occurs after exposure to UV irradiation. HEK293 cells were synchronized to S phase by double thymidine block and then irradiated with indicated dose of UV light. The PLA was performed as shown in (A). Nuclei were stained with Hoechst 33342. *P<0.05, **P<0.01. CTF18, chromosome transmission fidelity protein 18; RPA, replication protein A; PLA, proximity ligation assay; HU, hydroxyurea; UV, ultraviolet.

cleotide arms of the PLA probes creates a template for rolling circle amplification (RCA) only when the epitopes of the target proteins are in close proximity (<40 nm). Following amplification of the RCA, an oligonucleotide probe labeled with Texas Red fluorophore is added and hybridizes with the RCA product. All fluorescence data were obtained with a confocal microscope FV10i (Olympus Corporation, Tokyo, Japan) and z-stacked images (collected in 1 μm steps) were used for quantification of PLA signals with the Duolink Image Tool (version 1.0; Sigma-Aldrich). The PLA signals were calculated from three different fields and the scores were converted into fold change compared with the control.

Statistical analyses. All experiments were repeated in triplicate. Statistical significance for *in situ* PLA was determined by two-tailed unpaired Student's t-test using Graphpad Prism 5 (Graphpad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

CTF18 interacts with the RPA complex in HEK293 cells. To elucidate the mechanism underlying replication stress responses by CTF18-RFC, the present study aimed to identify a new binding partner for CTF18. It focused on a ssDNA-binding protein RPA that stabilizes the ssDNA region during DNA replication and repair, and also acts as a scaffold for DNA processing proteins. Although RPA is a heterotrimeric complex composed of 70, 32 and 14 kDa subunits, referred to as RPA1, RPA2 and RPA3, respectively (22), the present study investigated the interaction between endogenous CTF18 with two RPA subunits, RPA1 and RPA2, which are detectable with specific antibodies. As shown in Fig. 1, an immune-complex of CTF18 from whole cell lysates of HEK293 cells included RPA1 and RPA2, suggesting that CTF18 forms a complex with RPA *in vivo*.

CTF18 is associated with RPA in response to replication stress. If the interaction between CTF18 and RPA occurs in the

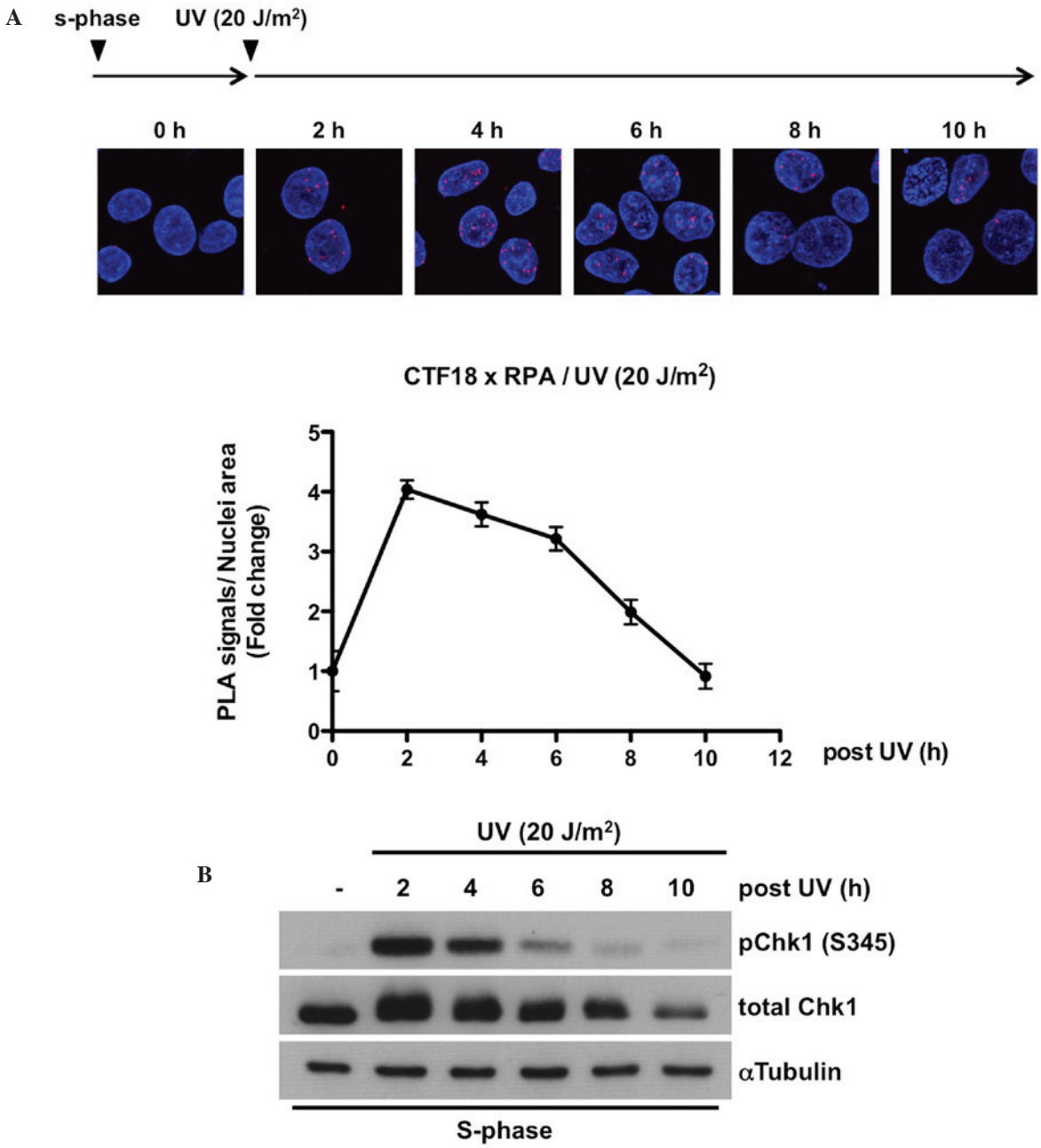

Figure 3. UV-induced CTF18-RPA interaction and Chk1 phosphorylation gradually decrease with time. (A) The dissociation kinetics of the CTF18-RPA complex in HEK293 cells. PLAs were performed with the indicated time course (magnification, x240). The Duolink Image Tool was used to quantify PLA signals. The vertical axis shows the total number of nuclear PLA signals divided from the nuclei and was normalized to non-irradiation group, and the horizontal axis indicates the time course after UV irradiation. Error bars indicate the standard error of the mean of three different fields. (B) The phosphorylation of Chk1 decreases after UV irradiation. HEK293 cells synchronized at S phase were irradiated with UV (20 J/m^2). After the indicated times, whole-cell lysates were immunoblotted with antibodies as indicated. Red fluorescent foci indicate the proximity of the two proteins and the nuclei were stainedwith Hoeschst 33342/ UV, ultraviolet; CTF18, chromosome transmission fidelity protein 18; Chk1, checkpoint kinase 1; RPA, replication protein A; PLA, proximity ligation assay; p-, phosphorylated.

replication stress responses, it is expected that the CTF18-RPA complexes could be observed in the nucleus when replication forks are stalled during S phase. To test this hypothesis, the PLA assay was conducted, where two endogenous proteins are immunostained with secondary antibodies, originating from different species, conjugated to complementary oligonucleotides. In this assay, when two distinct antibodies locate in close proximity (<40 nm), the conjugated DNA can be amplified and detected with a fluorescent probe as foci that represent molecules of each of two interacting proteins (23,24). To induce the replication checkpoint response, double-thymidine arrested HEK293 cells were released into S phase and subsequently treated with HU, which causes a reversible inhibition of DNA synthesis and thus blocks the progression of replication forks (25). The DuoLink assay showed that there is no significant signal in the absence of HU, whereas HU treatment resulted in the formation of nuclear PLA foci in a dose-dependent manner (Fig. 2A). These results suggest that the S-phase checkpoint response elicited by stalled replication forks leads to the interaction between CTF18 and RPA in the nucleus.

Interaction between CTF18 and RPA occurs after UV irradiation. In addition to the S-phase checkpoint pathway, eukaryotic cells can tolerate replication stress by bypassing DNA lesions via TLS (6). Since CTF18 has been shown to be implicated in TLD (11), it was investigated whether UV-induced DNA damage triggered CTF18-RPA interaction during S phase. Synchronized HEK293 cells at early S phase were exposed to UV irradiation at 20 or 100 J/m^2, and after 2 h, the CTF18-RPA interaction was assessed by counting PLA foci. A few foci were observed in the nucleus following exposure to 20 J/m^2 UV, and the formation of nuclear foci was significantly augmented when irradiated with a high dose (100 J/m^2) of UV light compared with a low dose (20 J/m^2; Fig. 2B). These findings suggest that the CTF18-RPA interaction occurs in response to the initiation of translesion DNA synthesis, although it is impossible to exclude the possibility that the interaction may result from the S-phase checkpoint response by UV irradiation.

Kinetics of the CTF18-RPA interaction correlate positively with that of Chk1 phosphorylation. Finally, it was hypothesized that if the CTF18-RPA interaction is required for the replication stress responses, it may be sustained until the attenuation of the S-phase checkpoint pathway. Therefore the dissociation kinetics of the CTF18-RPA complex were examined by tracking the time course of PLA signals and comparing it with the activation status of the ATR-Chk1 signaling pathway following UV-induced replication stress. As shown in Fig. 3A, it was demonstrated that while the number of foci peaks at 2 h after UV irradiation, it gradually decreases with time and almost disappears before 10 h. In addition, this time-dependent decline in UV-induced binding of CTF18 to RPA was similar to that of Chk1 phosphorylation at Ser345 (Fig. 3B). Collectively, these data imply that the CTF18-RPA interaction is involved in the replication stress response, including translesion DNA synthesis and S-phase checkpoint pathway.

Discussion

The present data demonstrated that RPA is a novel binding partner of CTF18 in mammalian cells. It was shown that this interaction was triggered when replication stress occurred and then gradually diminished in accordance with a decrease in the phosphorylation levels of Chk1 at Ser345. Accumulating evidence has shown that the CTF18-RFC complex is critical in activation of the S phase checkpoint and translesion DNA synthesis by interacting with DNA polymerase ε and η, respectively (10-13). However, the mechanism whereby CTF18-RFC responds to replication stress and targets the stalled replication forks remains to be determined. In this study, it was hypothesized that RPA may serve as a platform for the molecular assembly of CTF18-RFC together with DNA polymerase ε and η, which in turn aids in producing an efficient response to replication stress.

The *in situ* PLA demonstrated that replication stress induced by HU treatment or UV irradiation triggers the interaction between CTF18 and RPA in the nucleus. It remains to be determined how CTF18 senses replication stress and binds preferentially to RPA on ssDNA. A possible mechanism could be the phosphorylation of the RPA2 subunit in response to replication stress. Several studies have shown that stalled replication forks cause hyperphosphorylation of RPA2 at the N-terminal region through the DNA damage response pathways involving the ATR and the DNA-dependent protein kinase (21,26-28). Moreover, phosphorylation of RPA2 is known to prevent its association with the replication machinery and thus be considered as a trigger for redirecting RPA functions from DNA replication to DNA damage responses (28,29). In agreement with this, RPA2 phosphorylation has been reported to enhance its interactions with the ATR and the 9-1-1 checkpoint clamp (30,31). Hence, although further studies are required to address the link between RPA2 phosphorylation and CTF18-RPA interaction, the present results have provided insight into the molecular basis of the initiation of the replication stress response in mammalian cells.

Among the four clamp loader complexes, the Elg1-RFC is hypothesized to act principally as an unloader for PCNA from nascent DNA after the passage of replication forks and thereby regulate PCNA levels in chromatin (32-34). In addition, Bylund and Burgers (35) demonstrated that CTF18-RFC also unloads PCNA specifically when ssDNA is coated with RPA, and they proposed a model in which this unloading activity of CTF18-RFC may contribute to establishing sister chromatid cohesion. However, considering the present result that CTF18 binds to RPA after UV-irradiation during S phase, it is possible that CTF18-RFC may remove monoubiquitinated PCNA after replicative bypass of UV-induced CPD with Polh and subsequently reload unmodified PCNA to restart normal DNA replication. Thus, the present results provide insight into the mechanism how DNA polymerases switch during TLS.

In conclusion, the present study demonstrated that CTF18 forms a complex with RPA when replication stress is elicited by hydroxyurea treatment or UV exposure during S phase. The interaction kinetics between CTF18 and RPA is positively associated with the phosphorylation status of Chk1. These results suggest that RPA may be a scaffold for CTF18-RFC to be recruited to stalled replication forks and respond to replication stress.

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