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Replication-mediated disassociation of replication protein A–XPA complex upon DNA damage: implications for RPA handing off

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Abstract

RPA (replication protein A), the eukaryotic ssDNA (single-stranded DNA)-binding protein, participates in most cellular processes in response to genotoxic insults, such as NER (nucleotide excision repair), DNA, DSB (double-strand break) repair and activation of cell cycle checkpoint signalling. RPA interacts with XPA (xeroderma pigmentosum A) and functions in early stage of NER. We have shown that in cells the RPA–XPA complex disassociated upon exposure of cells to high dose of UV irradiation. The dissociation required replication stress and was partially attributed to tRPA hyperphosphorylation. Treatment of cells with CPT (camptothecin) and HU (hydroxyurea), which cause DSB DNA damage and replication fork collapse respectively and also leads to the disruption of RPA–XPA complex. Purified RPA and XPA were unable to form complex *in vitro* in the presence of ssDNA. We propose that the competition-based RPA switch among different DNA metabolic pathways regulates the dissociation of RPA with XPA in cells after DNA damage. The biological significances of RPA–XPA complex disruption in relation with checkpoint activation, DSB repair and RPA hyperphosphorylation are discussed.

Keywords

DNA repair; DNA damage; nucleotide excision repair; RPA; XPA

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Author contribution

Gaofeng Jiang and Xiaoming Wu performed all experiments and analysed data. Yue Zou and Xiaoming Wu designed experiments and wrote the paper.

1. Introduction

RPA (replication protein A) is an ssDNA (single-stranded DNA)-binding protein that plays fundamental roles in numerous DNA metabolic pathways. Human RPA is a multimeric complex that contains three tightly associated subunits of 70, 32 and 14 kDa (referred to hereafter as RPA70, RPA32 and RPA14 respectively). RPA was originally identified as a factor indispensable for *in vitro* SV40 (simian virus 40) DNA replication. It was subsequently found that RPA was required for almost all aspects of cellular DNA metabolism, including DNA replication, recombination and repair. RPA participates in these diverse pathways through its strong affinity for ssDNA and its ability to interact with a variety of protein partners (Wold, 1997; Iftode et al., 1999; Zou et al., 2006).

The involvement of RPA in eukaryotic NER (nucleotide excision repair) has been extensively studied (Costa et al., 2003; Thoma and Vasquez, 2003; Binz et al., 2004; Shuck et al., 2008). The heterotrimeric RPA forms a complex with XPA (xeroderma pigmentosum A) and functions at the early stage of human NER. The interaction between RPA and XPA appears to be crucial for the efficient NER action since deletion of the XPA-interacting domain of RPA32 or RPA70-interacting domain of XPA resulted in poor *in vitro* and *in vivo* NER activity (Li et al., 1995; Stigger et al., 1998). In the later stage of NER, RPA participates in the gap-filling reaction, along with PCNA (proliferating-cell nuclear antigen), RFC and DNA polymerase δ or ϵ (Wold, 1997; Iftode et al., 1999). RPA is also involved in recombinational repair of DNA DSB (double-strand break). Rad52 specifically recognizes RPA-bound ssDNA, which allows the Rad51–Rad52 complex to gain access to ssDNA already covered with RPA. RPA stimulates recombination reaction *in vitro*, especially when long DNA substrates are used (Park et al., 1996; Sugiyama and Kowalczykowski, 2002; Sung et al., 2003; Grimme et al., 2010). Furthermore, RPA-coated ssDNA has an essential role in the activation of DNA damage checkpoint (Zou and Elledge, 2003; Zou et al., 2003, 2006; Wu et al., 2005a; Choi et al., 2010; Liu et al., 2011).

Given that RPA functions in diverse pathways, whereas XPA has no known involvement except with NER, RPA–XPA interaction might be regulated *in vivo*. Indeed, RPA phosphorylation seems to modulate its interactions with proteins and DNA (Binz et al., 2003, 2004; Oakley et al., 2003). RPA32 is phosphorylated in a cell cycle-dependent manner (Binz et al., 2004), referred to hereafter as hypophosphorylation, to distinguish from damage-induced hyper-phosphorylation, and may further undergo hyperphosphorylation in response to various genotoxic agents, for example, UV or ionizing irradiation, HU (hydroxyurea) and CPT (camptothecin) (Binz et al., 2003, 2004). Damage-induced hyperphosphorylation of RPA32 seems to involve members of the PIKK (phosphatidylinositol 3-kinase-related kinase) family, including DNA-PK (DNA-dependent protein kinase), ATM (ataxiatelangetasia mutated) and ATR (ATM- and Rad3-related) (Binz et al., 2003; Zou et al., 2006). RPA32 hyperphosphorylation alters the conformation of the heterotrimer, which, consequently, affects its interactions with proteins and DNA (Binz et al., 2003, 2004; Oakley et al., 2003; Wu et al., 2005b). Thus hyperphosphorylation of RPA abolishes its interaction with DNA-PK and p53 (Abramova et al., 1997; Shao et al., 1999).

We report here that cellular RPA–XPA interaction is disrupted upon higher dose of UV irradiation, as well as by HU and CPT treatment. Disruption is dependent on replication stress and partly due to RPA hyperphosphorylation, as indicated by the greatly reduced interaction between XPA and hyperphosphorylated isoforms of RPA. In addition, we report that direct competition for RPA among different DNA processing events may also contribute to this disruption. We hypothesize that RPA, when dissociated with XPA, shifts from NER machinery to the pathways of DSB repair or checkpoint activation. The results support the notion that RPA plays a dynamic modulatory role *in vivo* to coordinate distinct cellular responses to DNA damage.

2. Materials and methods

2.1. Cell culture and treatments

Human lung adenocarcinoma cells, A549, were obtained from ATCC and maintained at 37°C and 5% CO₂ in DMEM (Dulbecco's modified Eagle's medium; Invitrogen) supplemented with 10% FBS (fetal bovine serum; Invitrogen) and 1% penicillin–streptomycin (Invitrogen). For UV exposure, the growth medium was removed and cells were washed once with PBS, and irradiated with various doses using a UV crosslinker (Stratagene) at 0.5 J·m⁻²·s⁻¹. After UV exposure, the original growth medium was removed, and the cells incubated for 2 h before collection. Chemicals used in this study were purchased from Sigma, unless otherwise stated.

2.2. PI (propidium iodide) staining

Cells were collected by centrifugation and fixed by adding 1 ml of ice-cold 70% ethanol drop-by-drop to the pellet. After being kept overnight at 4°C, the cells were washed thrice with PBS by centrifugation for 10 min at 3000 rpm. PI staining solution (0.5 ml, containing 50 µg/ml PI and 0.1% of RNase A in PBS buffer) was added and incubated at room temperature for 30 min in the dark. Samples were analysed on a FACScan flowcytometer.

2.3. Preparations of whole cell extracts and Western blotting

Cells were washed once with PBS, scrapped and collected in ice-cold PBS. After brief centrifugation at 12000 rpm, for 20 s, they were resuspended in solution A (50 mM Tris/HCl, pH 7.8, 400 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.34 M sucrose, 10% glycerol, 1 mM Na₃VO₄, 10 mM NaF, 2-glycerophosphate, 1 mM PMSF and protease inhibitor cocktail; Roche) and left on ice for 30 min to lyse. Lysates were cleared by centrifugation at 12000 rpm for 30 min at 4°C, and the protein concentration was determined by the Bradford assay (Bio-Rad). Lysates were separated on 8 or 12% (for RPA32) SDS-polyacrylamide gels and transferred to PVDF membranes (Amersham Biosciences). The membranes were blocked for 1 h at room temperature with TBST (25 mM Tris/HCl, pH 7.5, 150 mM NaCl and 0.05% Tween-20) containing 5% (w/v) non-fat dried skimmed milk powder and probed using following primary antibodies: anti-RPA32 (1:1000; Kamiya Biomedical Company) or anti-RPA70 (1:500; Santa Cruz Biotechnology). The membranes were incubated with HRP (horseradish peroxidase)-linked secondary anti-mouse antibodies (Amersham) and bound antibodies were visualized using ECL[®] chemiluminescent method (Amersham).

2.4. Co-IP (immunoprecipitation) assays

Whole cell extracts or nuclear extracts prepared from 1×10^7 cells were used for each co-IP reaction. Cell lysates were diluted with buffer (50 mM Tris/HCl, pH 7.8, 1 mM EDTA, 10% glycerol, protease and phosphatase inhibitor as above), and incubated with the indicated antibodies for 10–14 h at 4°C with end mixing. The following antibodies were used for IP assays: rabbit anti-XPA antibody (Santa Cruz Biotechnology), and rabbit anti-phospho-RPA32 Ser⁴/Ser⁸ antibody (Bethyl Laboratories). Protein A-agarose beads (50 µl; Amersham) were added and the reaction mixtures mixed for 1 h at 4°C. The immunoprecipitates were separated from the supernatant by centrifugation and washed with PBS containing 0.05% Nonidet P-40. Proteins were extracted from the agarose beads by boiling in 1×SDS gel loading buffer and resolved on 12% SDS-polyacrylamide gels.

2.5. Pull-down assays

Recombinant His-XPA was purified as described previously (Yang et al., 2002). For each binding assay, 2 µg of His-XPA was mixed with whole cell extracts prepared from 2×10^6 cells. The reaction mixtures were rotated at 4°C for 10–14 h and 50 µl Ni-NTA (Ni²⁺-nitrilotriacetate) resin (Qiagen) was added, followed by additional incubation at 4°C for 30 min. After extensive washes with PBS, bound RPA was detected by SDS/PAGE and immunoblotting with anti-RPA32 antibody.

2.6. GMSA (gel mobility shift assay)

Recombinant His-XPA and RPA were purified as described previously (Yang et al., 2002). GMSA was carried out to examine the effects of ssDNA on RPA–XPA complex formation. The two proteins and a [γ -³²P] ATP-labelled 50 nt oligonucleotide with a random sequence were mixed in two different orders to test the effects. In one case, RPA was incubated with increasing amounts of XPA at room temperature for 15 min, and 5 nM ssDNA added into the reaction and incubated for a further 15 min. In the other case, RPA was incubated with ssDNA, followed by increasing amounts of XPA. After reaction, 2 µl of 80% (v/v) glycerol was added, and the mixture immediately loaded on to a 4% native polyacrylamide gel in 1×TBE (Tris/borate/EDTA) running buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.3) for electrophoresis at room temperature. After drying the gels, the free and bound DNA were visualized using a PhosphorImager (FLA-5000, FUJIFILM).

2.7. Immunofluorescence

Cells were grown on 18 mm coverslips (Fisher Scientific) overnight prior to treatment. They were treated with the indicated dose of UV irradiation or sham treated. After 2 h, the cells were washed with PBS, extracted with PBS containing 0.5% NP-40 for 5 min on ice, and fixed with 100% methanol at –20°C for 10 min. They were blocked for 30 min in PBS containing 15% FBS. Primary antibody dilutions were: rabbit anti-phospho-RPA32 Ser⁴/Ser⁸ 1:2000 (Bethyl Laboratories), mouse anti-XPA 1:500 (Kamiya Biomedical Company), rabbit anti-XPA 1:500 (Santa Cruz), mouse anti-RPA32 1:250 (Kamiya Biomedical Company). Secondary antibody dilutions were: anti-rabbit Alexa Fluor[®] 488 1:250 and anti-mouse Alexa Fluor[®] 568 1:250 (Molecular Probes). Images were captured with a Nikon

inverted fluorescent microscope with attached CCD (charge-coupled-device) camera at $\times 100$ magnification and processed using Photoshop 6.0 (Adobe) software.

3. Results

3.1. UV irradiation induces the down-regulation of RPA–XPA interaction in cells

Although the interaction of RPA with XPA is well known, the potential regulation of this interaction in cells after DNA damage is yet to be investigated. Thus cells were treated with different doses of UV irradiation, and co-IP assays using anti-XPA antibody were performed to probe the cellular interaction between XPA and RPA. UV treatment did not cause significant cell death as shown by PI staining to detect apoptosis (Figure 1A). Similar to previous observations (Wu et al., 2005a, 2005b), treatment resulted in significant hyperphosphorylation of RPA32 subunit, as indicated by the appearance of additional slower-migrating bands on SDS/PAGE (Figure 1B, input). Interestingly, UV irradiation led to a dose-dependent ($5\text{--}20\text{ J/m}^2$) reduction in XPA co-precipitation with both subunits of RPA (Figures 1B and 1C). When UV dosage was increased to 40 J/m^2 , the interaction of RPA with XPA was completely disrupted (data not shown). Such a reduced association between XPA and RPA was also observed when recombinant *His*-XPA was applied to pull-down assays (Figure 1D). Notably, relatively less hyperphosphorylated isoforms of RPA (the slowest migrating forms indicated by the arrow) associated with XPA than the native RPA under the experimental conditions (Figure 1B). The reduced interaction between hyperphosphorylated RPA and XPA also occurred in an UV dose-dependent manner. Considerable interaction was observed at 5 J/m^2 of UV irradiation, whereas the interaction was significantly reduced at 10 J/m^2 and was barely detectable after 20 J/m^2 UV treatment (Figure 1B). In a reciprocal co-precipitation experiment, hyperphosphorylated RPA antibody also pulled down a reduced amount of XPA in an UV dose-dependent manner (Figure 1E). Moreover, the decreased association between XPA and hyperphosphorylated RPA was not due to altered intrinsic affinity between hyperphosphorylated RPA and XPA because purified RPA hyperphosphorylated by DNA-PK *in vitro* interacted with *His*-XPA as efficiently as native RPA in pull-down assays (our unpublished data, but see Patrick et al., 2005; Stigger et al., 1998). This suggests that some other pathways activated in an UV dose-dependent manner may preferentially recruit hyperphosphorylated RPA, and thus lead to disruption of the XPA interaction with hyperphosphorylated RPA. Since XPA undergoes post-translational modifications (Wu et al., 2006; Fan and Luo, 2010) in response to UV treatment, we investigated whether phosphorylation of XPA contributes to dissociation of the RPA–XPA complex. Cells were transfected with plasmid encoding a mutant XPA (XPA-S196A), in which Ser¹⁹⁶ of XPA was substituted by alanine, and subjected to UV irradiation. Co-IP experiments showed that the interaction between RPA and the phosphorylation-defective mutant XPA also decreased in response to UV treatment. Therefore the phosphorylation status of XPA did not affect the dissociation of RPA and XPA after UV treatment.

3.2. Disruption of RPA–XPA interaction in response to CPT and HU treatments

To explore the potential mechanisms underlying the disassociation of RPA and XPA in cells upon UV irradiation, the cells were treated with several different types of genotoxic agents.

Treatment with CPT, a radiomimetic agent, resulted in substantial hyperphosphorylation of the RPA32 subunit, and also the abrogation of the interaction between RPA and XPA (Figure 2A). The cells were also treated with HU, which causes no direct damage to DNA, but inhibits ribonucleotide reductase and leads to nucleotide depletion, thereby inducing replication forks collapse and cell cycle checkpoint activation. Likewise, HU caused the disruption of RPA–XPA complex formation (Figure 2B). Moreover, a low dose (1 mM) of HU treatment, which usually leads only to replication forks collapse, also caused apparent complex disruption (Figure 2C). Since CPT and HU treatment induce no DNA damage to the NER pathway, this suggests that RPA may be preferentially recruited to other cellular pathways after DNA damage, thereby precluding its association with XPA.

3.3. Disruption of RPA–XPA complex after UV irradiation requires replication stress

Since RPA is involved in DSB repair as well as DNA damage checkpoint signalling after DNA damage, we hypothesized that both pathways, where long stretches of ssDNA are generated, might sequester RPA from its association with XPA. DNA replication is required for activation of ATR-dependent DNA damage checkpoint (Lupardus et al., 2002), conversion of NER intermediates or unrepaired DNA lesions into DSBs (Dunkern and Kaina, 2002), and hyperphosphorylation of RPA32 (Rodrigo et al., 2000; Oakley et al., 2001). Thus, the dependency of RPA–XPA disruption on DNA replication was examined. Cells were synchronized in G₁ or S phase. After treatment with increasing doses of UV, the G₁ phase cells were found to contain only a small amount of hyperphosphorylated RPA32 (Figure 3A, upper panel). In contrast, a significant portion of RPA in S-phase cells was hyperphosphorylated as the UV dose increased indicating DNA replication is required for RPA hyperphosphorylation after UV treatment (Figure 3C, upper panel). Interestingly, after higher dose of UV treatment, no obvious disassociation of RPA–XPA complex was observed in G₁-phase cells (Figure 3A, lower panel), whereas synchronized S-phase cells exhibited significant RPA–XPA disruption (Figure 3C, lower panel). The inhibition of DSB formation in G₁-phase cells was verified by assessing the level of γ H2AX (phosphorylated histone H2AX; Redon et al., 2002), a marker of DSB formation (Figure 3D). To examine the requirement of DNA replication for disruption of RPA–XPA interaction after UV DNA damage, cells were treated with aphidicolin, a replicative polymerase inhibitor, to inhibit DNA replication. Aphidicolin treatment abolished the hyperphosphorylation of RPA after UV treatment (compare Figures 3B and 1B, input), and no obvious disruption of RPA–XPA interaction was observed (Figure 3B, lower panel). These results suggest that cellular DNA replication-induced DSB formation or checkpoint activation after UV irradiation plays a critical role in the regulation of RPA–XPA interaction, possibly through a competition-based mechanism to regulate RPA–XPA disruption.

3.4. RPA–XPA does not form a complex *in vitro* in the presence of ssDNA

To determine if the ssDNA generated in DSB repair or checkpoint activation processes in cells interferes with the interaction of XPA with RPA, an *in vitro* assay was developed to test the effects of ssDNA on RPA–XPA complex formation. As shown in Figure 4(A), in the absence of XPA, the ssDNA (50 nt) was bound by one RPA molecule at lower concentrations (lower band) and two RPA molecules at higher concentrations (upper band; lane 1–5), consistent with the notion that 30 nt are the occluded size for RPA binding (Lee et

al., 1995; Iftode et al., 1999). Interestingly, pre-incubation of RPA with excess amount of XPA did not change the RPA–ssDNA binding pattern; that is, no XPA-RPA–ssDNA supercomplex was formed (lanes 6–11, Figure 4A) (Lee et al., 1995). This suggests that XPA may not complex with RPA in the presence of ssDNA, although RPA and XPA have been well documented to form a complex *in vitro* (Yang et al., 2002). Similar observations were obtained when RPA and ssDNA were pre-mixed followed by the addition of XPA (Figure 4B).

3.5. Inefficient co-localization of XPA with hyperphosphorylated RPA in cells after UV irradiation

Nuclear co-localization of XPA with RPA and hyperphosphorylated RPA in cells following 15 J/m² of UV irradiation was examined. UV exposure triggered the formation of discrete RPA and XPA nuclear foci which, as expected, largely co-localized with each other, as probed by immunofluorescent staining with RPA32 antibody (which recognizes both native and hyperphosphorylated RPA) and the XPA antibody (Figure 5A). This suggests that after a moderate dose of UV irradiation, RPA interacts with XPA to participate in NER reaction. In contrast, only a very limited number of co-localized foci of hyperphosphorylated RPA and XPA were observed (Figure 5B), suggesting that hyperphosphorylated RPA may physically and functionally dissociate from XPA to perform other cellular functions. In support, hyperphosphorylated RPA co-localized with Rad52 and ATR efficiently after UV damage (Wu et al., 2005b).

4. Discussion

Cellular DNA is constantly under attack from DNA-damaging agents. In general, removal of most (if not all) types of DNA lesions relies on cellular DNA-repair mechanisms. To allow adequate time for the repair, cell cycle checkpoints have evolved to delay cell cycle transitions. As an essential component involved in almost all aspects of DNA metabolism, RPA likely plays a regulatory role in coordinating these pathways (Wold, 1997; Iftode et al., 1999; Zou et al., 2006). The results presented herein provide novel insight into the role of RPA in this regulatory process.

We found the XPA–RPA complex dissociated after several different genotoxic agents treatment. This was unexpected, since the interaction of XPA with RPA in NER process has been well established. So what is the mechanism by which the disassociation of RPA–XPA complex is induced in cells? First, RPA hyperphosphorylation appeared to play a role in this process because hyperphosphorylated RPA interacted and co-localized much less efficiently with XPA than native RPA in cells (Figures 1B and 5B). However, *in vitro* hyperphosphorylated RPA had a similar affinity to XPA as native RPA (Stigger et al., 1998; Patrick et al., 2005). Therefore it is plausible that other cellular pathways activated in DNA damage-dependent manner may specifically recruit hyperphosphorylated RPA. This hypothesis is likely true because hyperphosphorylated RPA interact preferentially with factors involved in DSB repair and DNA damage checkpoint activation pathways, such as Rad51, Rad52 and ATR (Shi et al., 2010; Wu et al., 2005b; Deng et al., 2009).

Secondly, the long length of ssDNA generated from a DNA DAB (diaminobenzidine) or DNA replication fork collapse may compete with XPA to interact with RPA. RPA, also termed ssDNA-binding protein, binds very tightly to ssDNA with an apparent association constant (K_a) of 10^8 – 10^{11} M⁻¹, depending on the length of DNA used (Wold, 1997; Iftode et al., 1999), while its affinity for XPA, measured by surface plasmon resonance, is 1.9×10^{-8} M (K_d) (Saijo et al., 1996). This indicates that ssDNA binds more competitively to RPA than XPA. Moreover, a recent NMR spectroscopy study showed the overlapping ssDNA- and XPA-binding sites on RPA70 subunit, suggesting that formation of RPA–XPA complex is modulated by ssDNA (Daughdrill et al., 2003). In close agreement with this notion, purified XPA and RPA were unable to form complex in the presence of ssDNA (Figures 4A and 4B). The inhibitory effect of ssDNA on RPA–p53 complex formation has also been reported (Miller et al., 1997). On the basis of these observations, we propose that in the cellular milieu, when sufficient amount or length of ssDNA accumulates as the result of replication fork collapse by non-repaired DNA lesions (or even ongoing repair assembly) and subsequent DSB homologous recombinational repair and nuclease attacks, ssDNA competitively sequester RPA and preclude its association with protein partners, such as XPA. The finding that CPT and HU treatments also lead to disassociation of RPA–XPA interaction supports this notion.

Thirdly, the competition between protein partners of RPA for binding to RPA, especially between XPA and Rad52, may also contribute to the dissociation of RPA–XPA complex. The structural study has defined a common surface on RPA for interaction with uracil-DNA glycosylase (UNG2), XPA and Rad52, each of which functions in a different DNA repair pathway, suggesting that the RPA interactions with the NER, BER (base excision repair) and recombinational repair machineries may be exclusionary (Mer et al., 2000; Jackson et al., 2002). As an increased interaction between RPA, particularly hyperphosphorylated RPA, and Rad52 has been observed after DNA damage (Wu et al., 2005b), it is likely that the competition-based mechanism shifts a fraction of the cellular pool of RPA from NER pathway to DSB repair pathway, thus leading to the disassociation of RPA–XPA interaction.

So what is the biological significance for damage-induced dissociation of RPA–XPA? As shown in Figure 4, interaction of XPA with RPA may be inhibited in the presence of ssDNA, whereas RPA-coated ssDNA serves as an excellent substrate for the recognition by ATR–ATRIP and Rad52 (Zou and Elledge, 2003; Zou et al., 2003; Grimme et al., 2010). This fact leads us to speculate that RPA when dissociated with XPA is redirected to stalled replication forks for checkpoint activation or DSB repair. In support of this notion, the interaction of RPA with DSB repair factors Rad51, Rad52 and Mre11/Rad50/Nbs1 complex, the checkpoint protein ATR and the Rad9/Rad1/Hus1 complex are enhanced in response to DNA damage (Robison et al., 2004; Wu et al., 2005a, 2005b). In addition, the inhibition of DSB formation and checkpoint activation by repressing DNA replication restored RPA–XPA interaction (Figure 3) further supporting that competition-based RPA handing-off exists in cells. We propose that there exists a dynamic RPA transition event among different DNA metabolic pathways in cells after UV irradiation (Wu et al., 2005b). At relatively low doses of UV irradiation, limited amount of UV lesions with little DSBs are generated in cells and, thus RPA is actively involved in the NER via interaction with XPA to remove

UV-induced DNA damages. If UV doses are further increased, cellular DNA will be extensively damaged and a considerable number of DSBs will be generated. DNA replication forks may also stall at the sites of DNA damage that have not been timely removed. Therefore RPA is largely recruited to the stalled replication forks for checkpoint activation and to DSB repair pathway which is very crucial for cell viability. Such recruitment may be facilitated by the hyperphosphorylation of RPA (Wu et al., 2005b).

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Abbreviations

ATM	ataxia-telangiectasia mutated
ATR	ATM- and Rad 3-related
CPT	camptothecin
DNA-PK	DNA-dependent protein kinase
DSB	double-strand break
FBS	fetal bovine serum
GMSA	gel mobility shift assay
HU	hydroxyurea
IP	immunoprecipitation
NER	nucleotide excision repair
Ni-NTA	Ni ²⁺ -nitrilotriacetate
PI	propidium iodide
RPA	replication protein A
ssDNA	single-stranded DNA
XPA	xeroderma pigmentosum A

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Figure 1. Dissociation of RPA–XPA complex in cells after UV irradiation

(A) Exponentially growing cells were treated with 20 J/m² UV irradiation or mock treated followed by a 2 h recovery. Cells were harvested and apoptosis was analysed by PI staining as described in the Materials and methods section. (B) Cells were treated with indicated doses of UV followed by a 2 h recovery. Total cellular lysates were prepared and incubated with anti-XPA antibody for co-IP assays. Proteins from the immunoprecipitates were detected by Western blotting using anti-RPA32 (IP-XPA, middle panel) antibody. Lower panel was the longer exposure of middle panel (10 and 20 J/m² UV irradiation). As controls, 10% of the total volumes of the whole cellular lysates used for the IP also were included (input, upper panel). (C) Co-IP experiments were performed as described in (B), expect that anti-RPA70 antibody was used for Western blotting detection. (D) Cells were treated as in (B), and the total cell lysates prepared were mixed with 2 µg of *His*-XPA for 10–14 h, followed by 30 min incubation with Ni-NTA resin. The RPA pulled-down by *His*-XPA was detected with anti-RPA70 antibody. (E) Cells were treated as in (B) and the total cell lysates were immunoprecipitated with anti-phospho-RPA32 Ser⁴/Ser⁸ antibody. Proteins from the immunoprecipitates were detected by Western blotting using anti-XPA antibody. (F) Cells were transfected with plasmid pcDNA3.1-*His*-XPA-S196A. At 2 days after transfection, cells were treated with UV as described above. Total cell lysates were incubated with Ni-NTA resin. The endogenous RPA pulled-down by *His*-XPA-S196A was detected by RPA antibody.

Figure 2. Disruption of RPA–XPA interaction in response to CPT and HU treatments

(A) Cells were treated with the indicated doses of CPT for 2 h. After treatment, cells were washed once with PBS, and incubated at 37°C for 2 h. Total cell lysates were prepared and co-IP assays with XPA antibody was performed as described in the Materials and methods section. (B) Cells were treated with the indicated doses of HU for 3 h, and total cell lysates were prepared and used for co-IP assays as above. (C) Cells were treated with 1 mM HU for 3 h, and the total cell lysates were prepared and used for co-IP assays as above.

Figure 3. Disruption of RPA–XPA interaction after UV irradiation requires replication stress
(A) Cells were synchronized in G₁ phase with double thymidine block. After different doses of UV treatment, cells were incubated for 2 h before collection. Whole cell lysates were prepared and detected directly by Western blotting analysis (upper: input) or used for co-IP assays with anti-XPA antibody as described in the Materials and methods section. **(B)** Cells were incubated with 6 μM aphidicolin for 30 min prior to the UV irradiation. After UV treatment, the cells were continuously incubated in the presence of 6 μM aphidicolin for another 2 h before harvest. Whole cell lysates were prepared for co-IP assays with anti-XPA antibody. **(C)** Synchronized S-phase cells were treated with increasing doses of UV irradiation, and total cell lysates were prepared for co-IP assays with anti-XPA antibody. **(D)** Synchronized G₁ phase and S-phase cells were treated with increasing doses of UV irradiation, and total cell lysates were prepared and probed with anti-γH2AX antibodies (Bethyl Laboratories).

Figure 4. XPA does not form a complex with RPA in the presence of ssDNA

GMSAs were performed as described in the Materials and methods section. **(A)** The indicated amount of native RPA was incubated with increasing concentrations of XPA first, followed by the addition of [γ - 32 P] ATP-labelled 50 nt ssDNA (lanes 6–11), or RPA–ssDNA-binding assays were performed in the absence of XPA (lanes 2–5). After reaction, the mixture was separated on a 4% native polyacrylamide gel and visualized using a PhosphorImager. The complexes formed and free ssDNA are indicated on the right. **(B)** The indicated amounts of RPA and ssDNA were incubated together first, and increasing amounts of XPA were added, followed by a second incubation. The complexes formed were examined on a 4% native polyacrylamide gel.

Figure 5. Co-localization of XPA with RPA and hyperphosphorylated RPA after UV treatment in cells

Cells were mock-treated or treated with 15 J/m² UV irradiation followed by 2 h recovery. After extraction of cytoplasmic proteins, cells were fixed and incubated with primary and secondary antibodies, and visualized by fluorescent microscopy. **(A)** Cells were stained with rabbit anti-XPA antibody (green, subpanels C and G) and mouse anti-RPA32 antibody (red, subpanels B and F). Subpanels D and H are the merged images of the anti-XPA and anti-RPA32 stained cells. Subpanels A and E are the DAPI (4',6-diamidino-2-phenylindole)-stained nuclei. **(B)** Cells were stained with mouse anti-XPA antibody (red, subpanels B and F) and rabbit anti-phospho-RPA32 Ser⁴/Ser⁸ antibody (green, subpanels C and G). Subpanels D and H are the merged images of the anti-XPA and anti-phospho-RPA32 Ser⁴/Ser⁸ stained cells.