Replication protein A promotes 5′→3′ end processing during homology-dependent DNA double-strand break repair

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R eplication protein A (RPA), the eukaryotic single-strand deoxyribonucleic acid (DNA [ss-DNA])–binding protein, is involved in DNA replication, nucleotide damage repair, mismatch repair, and DNA damage checkpoint response, but its function in DNA double-strand break (DSB) repair is poorly understood. We investigated the function of RPA in homology-dependent DSB repair using Xenopus laevis nucleoplasmic extracts as a model system. We found that RPA is required for single-strand annealing, one of the homology-dependent DSB repair pathways. Furthermore, RPA promotes the generation of 3′ single-strand tails (ss-tails) by stimulating both the Xenopus Werner syndrome protein (xWRN)–mediated unwinding of DNA ends and the subsequent Xenopus DNA2 (xDNA2)–mediated degradation of the 5′ ss-tail. Purified xWRN, xDNA2, and RPA are sufficient to carry out the 5′-strand resection of DNA that carries a 3′ ss-tail. These results provide strong biochemical evidence to link RPA to a specific DSB repair pathway and reveal a novel function of RPA in the generation of 3′ ss-DNA for homology-dependent DSB repair.

Introduction

DNA double-strand breaks (DSBs) represent the most deleterious threat to genome stability. If not properly repaired, DSBs often lead to chromosome deletions or translocations and, consequently, premature cell death or oncogenic transformation (Vilenchik and Knudson, 2003). Three major pathways have been identified to repair DSBs: nonhomologous end joining (NHEJ), homologous recombination (HR), and single-strand annealing (SSA; Baumann and West, 1998; Karran, 2000; Pastink et al., 2001). NHEJ usually polishes and then directly joins DNA ends in an error-prone process. HR repairs DSBs by copying the missing information from a homologous sequence, which is usually the sister chromatid in mitotic cells. SSA can repair a break that occurs between two direct repeats, and the final product effectively retains only one of the two repeats. HR and SSA are both homology based and require the processing of DSB ends into 3′ single-strand tails (ss-tails; Symington, 2002).

In HR, the 3′ ss-tail invades the homologous chromosome, whereas in SSA the 3′ ss-tails from the two sides of the break anneal with each other.

Although the general scheme of the major DSB repair pathways has been outlined, many fundamental mechanistic questions remain poorly understood. For example, many human disease proteins, such as Brca1 and Brca2, have been implicated in DSB repair, but their exact mechanistic roles are still ambiguous despite intensive research. Another protein of great importance and the focus of this study is replication protein A (RPA), the eukaryotic single-strand DNA (ss-DNA)–binding protein (SSB; Wold, 1997). Through both ss-DNA binding and specific protein–protein interactions, RPA has been shown to participate in DNA replication, nucleotide excision repair, base excision repair, mismatch repair, and the ataxia telangiectasia and Rad3 related (ATR)–mediated checkpoint activation (Fanning et al., 2006). There is also evidence for RPA to function in DSB repair, in particular homology-dependent DSB repair. RPA interacts with recombination protein RAD51 and promotes the
coating of RAD51 onto ss-DNA and strand invasion (Golub et al., 1998; Stauffer and Chazin, 2004; Wang and Haber, 2004). It also interacts with RAD52 and promotes the complementary-strand annealing activity and repair center formation of RAD52 and HR (Mortensen et al., 1996; Park et al., 1996; Sung, 1997; Hays et al., 1998; Shinohara et al., 1998; Sugiyama et al., 1998; Plate et al., 2008). Genetic analyses have suggested that RPA participates in homology-dependent repair between direct repeats, gene conversion, and SSA, but the effect can be either stimulatory or suppressive depending on allele and assay (Firmenich et al., 1995; Smith and Rothstein, 1995; Hays et al., 1998; Umezu et al., 1998). Knockdown of RPA by siRNAs in mammalian cells also suggests that RPA plays an important role in homology-dependent DSB repair (Sleeth et al., 2007). However, the fact that RPA participates in so many DNA transactions complicates a rigorous mechanistic dissection of its role in DSB repair.

Like other biological processes, a thorough understanding of DSB repair should benefit greatly from in vitro systems that can reconstitute the various repair pathways. One powerful in vitro system is the extract derived from the eggs of the frog *Xenopus laevis*. This system can efficiently join various DNA ends via a Ku-dependent NHEJ mechanism (Thode et al., 1990; Labhart, 1999). A derivative extract, prepared from nuclei reconstituted in total egg extract and, thus, called nucleoplasmic extract (NPE), can efficiently reconstitute SSA (Yan et al., 2005). More recently, we have further explored NPE for studying the processing of double-strand DNA (ds-DNA) ends, which is the first reaction for both SSA and HR (Toczykowski and Yan, 2006). We have found that ds-DNA ends are degraded in an ATP-dependent manner and in the 5′→3′ direction to generate 3′ ss-tails, as expected of homology-dependent DSB repair pathways. A major (but not the only) pathway can be divided into two steps: the ATP-dependent unwinding of ds-DNA ends and the ATP-independent 5′→3′ degradation of ss-DNA tails. Moreover, we have found that the major helicase for end unwinding is the *Xenopus* Werner syndrome protein (WRN [xWRN]), whereas a major 5′→3′ single-strand exonuclease is the *Xenopus* DNA2 (xDNA2; Toczykowski and Yan, 2006; Liao et al., 2008; Wawrousek et al., 2010). This mechanism is remarkably similar to the one suggested for the *Escherichia coli* RecQ helicase and RecJ 5′→3′ ss-DNA exonuclease (Handa et al., 2009). It is also supported by many observations in yeast and mammalian cells. In budding yeast *Saccharomyces cerevisiae*, the RecQ-type helicase SGS1 and nuclease DNA2 and EXO1 participate in the 5′→3′ strand processing of DSBs (Mimitou and Symington, 2008; Zhu et al., 2008; Budd and Campbell, 2009). In mammalian cells, the Bloom syndrome protein (BLM), another RecQ-type helicase, and EXO1 act in parallel pathways to promote end processing (Gravel et al., 2008). Studies in *S. cerevisiae* further suggest that these two pathways act downstream of MRX (MRE11-RAD50-XRS2) and Sae2 (Mimitou and Symington, 2008; Zhu et al., 2008). Notably, homologues of MRX (MRN [MRE11-RAD50-NBS1]) and Sae2 (C-terminal–binding protein–interacting protein [CtIP]) are also important for strand resection in mammalian cells (Jazayeri et al., 2005; Sartori et al., 2007; Chen et al., 2008) and *Xenopus* egg extracts (You et al., 2009; Wawrousek et al., 2010). Collectively, these studies suggest that the mechanism for 5′-strand resection at DSBs is highly conserved in yeast, *Xenopus*, and mammals.

The helicase activity of xWRN and the 5′→3′ ss-DNA exonuclease activity of xDNA2 are both dramatically stimulated by RPA (Chen et al., 2001; Liao et al., 2008). This raises an interesting question about RPA’s own role in homology-dependent DSB repair and 5′-strand degradation. In this study, we use *Xenopus* egg extracts and biochemical characterizations of the relevant purified proteins to investigate these questions. We find that RPA is important for the SSA pathway of DSB repair. Moreover, we find that RPA is important for the degradation of the 5′ strand to generate the 3′ ss-tail. At the mechanistic level, RPA stimulates both the xWRN-mediated unwinding of ds-DNA ends and the subsequent xDNA2-mediated 5′→3′ degradation of ss-DNA. Furthermore, purified xWRN, xDNA2, and RPA are sufficient to degrade DNA that carries a 3′ ss-tail. These results not only provide the first biochemical evidence to link RPA to a specific homology-based DSB repair pathway in an in vitro system that fully reconstitutes SSA but also increase our understanding of the mechanism of 5′-strand degradation at DNA ends.

**Results**

**RPA is required for SSA**

We first analyzed the effect of RPA on the homology-dependent SSA pathway of DSB repair. SSA can be efficiently reconstituted in NPE with a linear DNA carrying two direct repeats (Yan et al., 2005). Specific anti-RPA antibodies were used to deplete it from NPE to a level below detection (>99% depletion; Fig. 1 A). The substrate, pRW4*, was a 5.6-kb linear DNA with a 1.2-kb Tet gene on each end (and the two cohesive ends were partially filled in with TTP and deoxy-CTP (dCTP) to block the simple religation of cohesive ends; Yan et al., 2005). In mock-depleted NPE, pRW4* was efficiently repaired by SSA (and NHEJ) into a series of products that increased in size over time as expected (Fig. 1 C, lanes 7–9). SSA was mostly an intermolecular reaction, and the major products were a 10-kb linear DNA (Fig. 1 C, arrow) and higher molecular weight bands (Fig. 1 C, bracket). The 10-kb DNA is formed from two pRW4* with one of the two junctions repeats effectively deleted. NHEJ gave rise to three products (Fig. 1 C, asterisks), corresponding to supercoiled circular monomers, relaxed circular monomers, and linear dimers. The higher molecular weight products are linear arrays formed by the continued addition of pRW4*, mostly through SSA. As shown in Fig. 1 C (lanes 4–6), RPA depletion caused a dramatic reduction in the formation of the SSA products. The formation of NHEJ products, in contrast, was little affected. To ensure that this effect was specific, we added the purified RPA back to the RPA-depleted NPE to determine whether it can complement the defect. As shown in Fig. 1 C (lanes 1–3), in the presence of the purified RPA, SSA products were again easily detected (the less than complete rescue was largely caused by the technical difficulty in adding a sufficient amount of RPA to the depleted NPE without over diluting it). Together, these data indicate that RPA is indeed required for the SSA pathway of DSB repair in NPE.
RPA is important for DNA end processing
What might be the mechanistic role of RPA in SSA? SSA is initiated by the 5′→3′ processing of ends to generate 3′ ss-tails, which are then annealed, and the resulting gaps and flaps are finally repaired. The function of RPA in complementary-strand annealing and DNA synthesis to fill in the gap is well established, so we focused on the potential effect of RPA on the 5′→3′ processing of ends. NPE contains robust activity for the 5′→3′ processing of ds-DNA ends (Toczylowski and Yan, 2006). The DNA substrate for end processing was prepared by linearizing pUC19 plasmid with EcoRI and then filling in the 3′ recessive ends with [32P]dATP and dideoxy-TTP (ddTTP). This DNA could not engage in either SSA because of the lack of homologous sequences at ends or NHEJ because of the block of ends by a dideoxynucleotide (Yan et al., 2005). When incubated in the mock-depleted NPE, the DNA was gradually degraded as judged by both SYBR gold DNA staining and 32P label (Fig. 2 A). However, in RPA-depleted NPE, the DNA was much more stable. Moreover, this defect could be efficiently complemented by the addition of the purified RPA protein (Fig. 2 A). Notably, the purified RPA protein by itself showed no nuclease activity, indicating that the complementation was not caused by the inadvertent introduction of some contaminating nuclease into the RPA-depleted NPE. An unrelated SSB, the phage T4 gp32 protein, also provided some complementation but was much less efficient than RPA in supporting end processing (Fig. 2 B). The molar amount of gp32 used was four times that of RPA and, thus, sufficient to bind approximately the same amount of ss-DNA as RPA could (Chen et al., 2001). Collectively, these data suggest that RPA stimulates the 5′→3′ processing of DNA ends and does so by mechanisms that appear to involve not only the stabilization of ss-DNA by the coating of SSBs but also the physical interactions between RPA and other end processing proteins.

RPA stimulates the xWRN-mediated unwinding of DNA ends
What might be the mechanistic role for RPA in DNA end processing? We have previously shown that a major (but not the only) end processing pathway in NPE can be divided into at least two steps: the unwinding of ds-DNA ends and the 5′→3′ degradation of the resulting ss-tails (Toczylowski and Yan, 2006). The major helicase for end unwinding is xWRN (Toczylowski and Yan, 2006). RPA is known to interact physically with xWRN and stimulates its helicase activity (Chen et al., 2001), suggesting that it might stimulate the unwinding step of end processing. We tested this hypothesis by using an assay depicted in Fig. 3 A. The substrate for the unwinding assay was a 48-bp oligonucleotide duplex (thio 5′ duplex). One strand carried a biotin moiety at its 5′ end, and the complementary strand carried 24 normal nucleotides in the 5′ half followed by 21 thionucleotides in the 3′ half.
We have previously shown that the 5’→3’ ss-DNA exonuclease activity of xDNA2 is dramatically stimulated by RPA (Liao et al., 2008). The substrate used in the study, 48mer-1, might form secondary structures, so it is possible that the role of RPA is simply to keep 48mer-1 in a single-strand state. To test this hypothesis, we also designed an oligonucleotide composed mostly of deoxyadenines (dAs; 48mer-5), which was predicted to be incapable of forming stable secondary structures at room temperature (Zuker, 2003). The two single-strand oligonucleotide substrates carried [32P]dA TPs near the 3’ end. The DNA was first coated onto Streptavidin magnetic beads and then incubated in either RPA- or mock-depleted NPE. End processing could only proceed from the 5’ end of the thio-containing strand and then stall at the thionucleotides (Toczylowski and Yan, 2006). If there had been no unwinding, the thio strand would have remained annealed to the biotin strand and, thus, bound to the beads. If there had been unwinding, the thio strand would have been released into the supernatant. As expected, in mock-depleted NPE, the partially degraded thio strand was released into the supernatant (Fig. 3 B). In RPA-depleted NPE, in contrast, a very little amount of the thio strand was released into the supernatant. This defect could be rescued by the purified RPA protein (Fig. 3 C). The T4 gp32 protein was, in contrast, much less efficient in supporting end unwinding (Fig. 3 D). These data suggest that RPA is required for efficient stimulation of the xWRN-mediated unwinding of DNA ends.

**RPA physically interacts with xDNA2 and stimulates the 5’→3’ ss-DNA exonuclease activity of xDNA2**

After the unwinding of DNA ends, the 5’ ss-tail is then degraded by the 5’→3’ single-strand exonuclease activity of xDNA2. We have previously shown that the 5’→3’ ss-DNA exonuclease activity of xDNA2 is dramatically stimulated by RPA (Liao et al., 2008). The substrate used in the study, 48mer-1, might form secondary structures, so it is possible that the role of RPA is simply to keep 48mer-1 in a single-strand state. To test this hypothesis, we also designed an oligonucleotide composed mostly of deoxyadenines (dAs; 48mer-5), which was predicted to be incapable of forming stable secondary structures at room temperature (Zuker, 2003). The two single-strand oligonucleotide substrates carried [32P]labeled dA (at positions 46 and 47 in 48mer-1 and at 20 potential positions distributed between position 22 and position 47 in 48mer-5) and bound to Streptavidin paramagnetic beads via the 3’-terminal biotin-deoxycytidine (dC; see Materials and methods). As shown in Fig. 4 A, with 48mer-1, xDNA2’s 5’→3’ nuclease activity was greatly stimulated by RPA, confirming our previous observation (Liao et al., 2008). The degradation stalled 12–17 nt away from beads, presumably because of steric hindrance. 48mer-5 showed slightly more degradation than 48mer-1 in the absence of RPA, suggesting that the lack of secondary structures does improve degradation by the intrinsic nuclease activity of xDNA2. However, in the presence of RPA, xDNA2’s nuclease activity was still...
To address this question, we prepared an ss-DNA substrate by heat denaturing the 3′-labeled linear ds-pUC19 DNA. We have previously shown that ss-DNA was degraded in NPE by 5′→3′ ss-DNA exonucleases, which were mainly xDNA2 (Liao et al., 2008). The denatured ss-DNA was incubated in RPA- or mock-depleted NPE and then analyzed by agarose gel electrophoresis. As shown in Fig. 5 A, although the ss-DNA was rapidly degraded in mock-depleted NPE, it was very stable in RPA-depleted NPE. This effect could be complemented by the purified RPA protein. Interestingly, the mobility of DNA was altered after incubation in RPA-depleted NPE in a way suggesting that most of the ss-DNA had been reannealed into ds-DNA, presumably by the strand-annealing proteins in the extract. RPA might thus facilitate ss-DNA degradation simply by preventing the single strands from reannealing. To test this hypothesis, we determined whether the T4 gp32 protein could substitute for RPA in ss-DNA degradation. As shown in Fig. 5 B, gp32 indeed led to a strong inhibition of strand reannealing, but the ss-DNA was degraded much less efficiently than in the control-depleted extract. Collectively, these data indicate that RPA plays an important role in the 5′→3′ ss-DNA degradation step of end processing. They also suggest that RPA does so both by physically stimulating the ss-DNA exonuclease activity of xDNA2 and by preventing the reannealing of ss-DNA strands.

xWRN, xDNA2, and RPA are sufficient to degrade DNA that carries a 3′ ss-tail

Is the stimulation of xDNA2’s ss-DNA exonuclease activity by RPA relevant to the degradation of 5′ ss-DNA tails in NPE?
incubated with xWRN, xDNA2, RPA, and ATP, it was efficiently processed (Fig. 6). All four components were required for this reaction. In particular, RPA could not be replaced by gp32, again confirming that RPA’s activity in end processing depends on its interaction with xWRN and xDNA2. In the absence of xDNA2, the preprocessed DNA was still gradually unwound, but not degraded, by xWRN and RPA. In the absence of RPA, DNA was stable except that the 3'→5' label was gradually lost, which was consistent with the weak intrinsic 3'→5' exonuclease of xWRN (Fry, 2002). Together, these results demonstrate that xWRN, xDNA2, and RPA are not only important but also sufficient for the 5'→3' degradation of preprocessed DNA.

As a 3'→5' DNA helicase, xWRN is incapable of initiating DNA unwinding from blunt ends (Fry, 2002). Indeed, when xWRN, xDNA2, and RPA are incubated with blunt-ended linear DNA, only trace amounts of DNA degradation could be detected (Fig. 6). Studies in yeast have suggested that Sgs1 acts downstream of MRE11 and/or Sae2, which appear to process DNA by unknown mechanisms to a limited extent to provide a 3' ss-tail for the more extensive processing by Sgs1 and DNA2 (Mimitou and Symington, 2008; Zhu et al., 2008). To test this hypothesis, we prepared a DNA substrate with preformed 3' ss-tails by limited digestion of the blunt-ended DNA with λ exonuclease. When such preprocessed DNA (carrying ~200–750 nt of 3' ss-DNA) exonuclease was incubated with xWRN, xDNA2, RPA, and ATP, it was efficiently processed (Fig. 6). All four components were required for this reaction. In particular, RPA could not be replaced by gp32, again confirming that RPA’s activity in end processing depends on its interaction with xWRN and xDNA2. In the absence of xDNA2, the preprocessed DNA was still gradually unwound, but not degraded, by xWRN and RPA. In the absence of RPA, DNA was stable except that the 3' label was gradually lost, which was consistent with the weak intrinsic 3'→5' exonuclease of xWRN (Fry, 2002). Together, these results demonstrate that xWRN, xDNA2, and RPA are not only important but also sufficient for the 5'→3' degradation of preprocessed DNA.
and xDNA2, which is involved exclusively in the 5' ss-tail degradation step (Liao et al., 2008). RPA's role in end unwinding is by stimulating the helicase activity of xWRN. It has long been observed that RPA can physically interact with WRN and stimulate its helicase activity, but the biological significance of this interaction has been unclear (Shen et al., 1998; Brosh et al., 1999; Chen et al., 2001). Our observation that RPA stimulates the xWRN-mediated unwinding of DNA ends for homology-dependent DSB repair provides a rationale for this interaction. RPA can also physically interact with xDNA2 and stimulates its 5'→3' ss-DNA exonuclease activity. Its role in 5' ss-tail degradation is thus most likely by stimulating the exonuclease activity of xDNA2. In addition to these two active roles mediated by protein–protein interaction, RPA also possesses a passive role in preventing the reannealing of ss-DNA. This passive role can be substituted by an unrelated SSB, such as the T4 gp32 protein, but the two active roles in stimulating the helicase activity of xWRN and the nuclease activity of xDNA2 cannot. The coupled mechanism of 5'-strand resection thus most likely depends on RPA to physically interact and stimulate both the xWRN-mediated end unwinding step and the xDNA2-mediated 5' ss-tail degradation step. These three proteins form a unique module to catalyze the 5'→3' processing of DNA ends for homology-dependent DSB repair. Interestingly, the E. coli RecQ helicase and RecJ nuclease are also stimulated by SSBs, implying that SSBs might play similar stimulatory roles in this 5'-strand resection pathway (Harmon and Kowalczykowski, 2001; Han et al., 2006; Shereda et al., 2007). Similarly, BLM, another RecQ helicase that has recently been shown to participate in end

Discussion

In this study, we use the Xenopus extract system and enzymatic characterizations to investigate the role of RPA in DSB repair. The major findings are that (a) RPA is required for SSA repair; (b) RPA is important for the 5'→3' strand-specific degradation at DNA ends; (c) RPA interacts with xDNA2 and stimulates its 5'→3' ss-DNA exonuclease activity; (d) RPA promotes both the xWRN-mediated unwinding of ends and the subsequent xDNA2-mediated degradation of 5' ss-tails; (e) RPA's function in 5'-strand processing cannot be fully replaced by the T4 gp32 SSB; and (f) xWRN, xDNA2, and RPA are sufficient to degrade the 5' strand of a 5' preprocessed DNA. As the major SSB in eukaryotic cells, RPA participates in probably every DNA transaction that involves ss-DNA. It has thus been difficult to rigorously determine its role in a particular pathway without concerns over indirect effects from defects in other processes, such as cell cycle progression or transcriptional regulation. Our study using the Xenopus system, which does not suffer from this drawback, provides direct biochemical evidence to link RPA to a specific DSB repair pathway. Moreover, it reveals a novel role for RPA in stimulating the 5'→3' processing of DNA ends to generate 3' ss-tails.

The processing of ds-DNA ends into 3' ss-tails is the first step of homology-dependent DSB repair. RPA is important for the 5' strand–specific degradation of DNA ends and does so by promoting both the unwinding of ends and the degradation of 5' ss-tails. This is in contrast to xWRN, which is involved exclusively in the end unwinding step (Toczylowski and Yan, 2006) and xDNA2, which is involved exclusively in the 5' ss-tail degradation step (Liao et al., 2008). RPA's role in end unwinding is by stimulating the helicase activity of xWRN. It has long been observed that RPA can physically interact with WRN and stimulate its helicase activity, but the biological significance of this interaction has been unclear (Shen et al., 1998; Brosh et al., 1999; Chen et al., 2001). Our observation that RPA stimulates the xWRN-mediated unwinding of DNA ends for homology-dependent DSB repair provides a rationale for this interaction. RPA can also physically interact with xDNA2 and stimulates its 5'→3' ss-DNA exonuclease activity. Its role in 5' ss-tail degradation is thus most likely by stimulating the exonuclease activity of xDNA2. In addition to these two active roles mediated by protein–protein interaction, RPA also possesses a passive role in preventing the reannealing of ss-DNA. This passive role can be substituted by an unrelated SSB, such as the T4 gp32 protein, but the two active roles in stimulating the helicase activity of xWRN and the nuclease activity of xDNA2 cannot. The coupled mechanism of 5'-strand resection thus most likely depends on RPA to physically interact and stimulate both the xWRN-mediated end unwinding step and the xDNA2-mediated 5' ss-tail degradation step. These three proteins form a unique module to catalyze the 5'→3' processing of DNA ends for homology-dependent DSB repair. Interestingly, the E. coli RecQ helicase and RecJ nuclease are also stimulated by SSBs, implying that SSBs might play similar stimulatory roles in this 5'-strand resection pathway (Harmon and Kowalczykowski, 2001; Han et al., 2006; Shereda et al., 2007). Similarly, BLM, another RecQ helicase that has recently been shown to participate in end
can also promote some limited end processing in the absence of Sgs1 and EXO1. Consistent with these genetic observations, depletions of neither xWRN nor xDNA2 can completely abolish end processing in \textit{Xenopus} extracts (Toczylowski and Yan, 2006; Liao et al., 2008; Wawrousek et al., 2010). Our reconstitution experiment showed that xWRN, xDNA2, and RPA cannot degrade a blunt-ended DNA. In addition, \textit{Xenopus} homologues of MRX (xMRN) and Sae2 (xCtIP) are also important for the formation of ss-DNA in \textit{Xenopus} egg extracts (You et al., 2009; Wawrousek et al., 2010). Further studies are required to rigorously test how these other factors/pathways are affected by RPA.

Are the in vitro biochemical experiments relevant to DNA end processing on chromosomal DNA in cells? An essential function for RPA in SSA is consistent with genetic analyses in \textit{S. cerevisiae}. For example, SSA has been characterized in the rfa1-t11 allele and shown to be 8.5-fold less efficient when compared with the wild-type strain. Another allele, rfa1-M2, displays a decreased rate of recombination.

Although gp32 cannot substitute for RPA to stimulate the purified xWRN and xDNA2, it does provide a partial complementation of end processing in RPA-depleted NPE. This suggests that there might be other end processing factors/pathways that depend on the stabilization of ss-DNA by an SSB, such as RPA or gp32, but do not require physical interactions with RPA. Genetic analyses in yeast \textit{S. cerevisiae} suggest that EXO1 appears to act in a pathway parallel to Sgs1 and that MRX and Sae2 can also promote some limited end processing in the absence of Sgs1 and EXO1. Consistent with these genetic observations, depletions of neither xWRN nor xDNA2 can completely abolish end processing in \textit{Xenopus} extracts (Toczylowski and Yan, 2006; Liao et al., 2008; Wawrousek et al., 2010). Our reconstitution experiment showed that xWRN, xDNA2, and RPA cannot degrade a blunt-ended DNA. In addition, \textit{Xenopus} homologues of MRX (xMRN) and Sae2 (xCtIP) are also important for the formation of ss-DNA in \textit{Xenopus} egg extracts (You et al., 2009; Wawrousek et al., 2010). Further studies are required to rigorously test how these other factors/pathways are affected by RPA.
Materials and methods

Extract preparation and nuclear reconstitution
Crude interphase extracts, membrane-free cytosol, and demembranated sperm chromatin were prepared according to the published procedures (Smythe and Newport, 1991). NPEs were prepared according to the published protocol (Walter et al., 1998).

Antibody preparation
The following antibodies were used in this study: rabbit anti-RPA, rat anti-RPA1 (the p70 subunit of Xenopus RPA), and rabbit anti-xWRN (amino acids 1–466). The rabbit anti-RPA antibodies were against all three subunits of the native RPA purified from Xenopus egg extracts (Fang and Newport, 1993) and used without further purification. The other two antibodies were raised against the corresponding recombinant GST fusion proteins according to the standard procedure (Goding, 1986). The antibodies were purified on affinity columns constructed with the corresponding recombinant proteins according to a procedure described previously (Yan et al., 1993).

Immunodepletion
Immunodepletion of cytosol was performed by incubating cytosol (40 + 20 µl ELB [10 mM HEPES, pH 7.5, 250 mM sucrose, 2.5 mM MgCl₂, 50 mM KCl, and 1 mM DTT]) with 20 µl protein A–Sepharose beads (Sigma-Aldrich) that had been precoated with rabbit anti-RPA serum or control serum (30 µl of serum/20 µl of beads). After incubation (with rotation) at 4°C for 2.5 h, the beads were removed by low speed centrifugation, and the supernatants were treated again with fresh beads. Immunodepletion of NPE was performed in a similar way except that the beads were coated with 40 µl of serum/20 µl of beads for a total of three rounds.

Interaction between RPA and xDNA2
For the communoprecipitation experiment, 10 µl protein A–Sepharose beads coated with 20 µl anti-xDNA2, anti-xRPA, and control sera was incubated with 30 µl cytosol (diluted 1.5× with ELB) at 4°C for 1 h. The beads were washed successively with 500 µl ELB, 500 µl ELB + 50 mM NaCl + 0.1% NP-40, and 500 µl ELB. The bound proteins were separated on an 8% SDS-PAGE, transferred to a polyvinylidene fluoride membrane, and probed for RPA, xDNA2, and xWRN by Western blotting. Detection was achieved with chemiluminescence (SuperSignal; Thermo Fisher Scientific).

To detect the interaction between the purified RPA and recombinant xDNA2, the full-length xDNA2 ORF was cloned by PCR using primers derived from the sequence in the database (Liu et al., 2000). After confirmation by sequencing, the xDNA2 ORF was subcloned into pFastBac (Invitrogen) to create a fusion protein with a FLAG tag at the C terminus. SFP cells expressing the recombinant xDNA2 protein were collected, and nuclear extracts were prepared according to the manufacturer’s instructions (Invitrogen). The extracted proteins were fractionated sequentially by HiTrap Q (peak at 250 mM NaCl) and HiTrap Heparin (peak at 425 mM NaCl) columns. 50 µl of the peak heparin fraction containing recombinant xDNA2 or 5 µg BSA (New England Biolabs, Inc.) was coated onto 10 µl anti-FLAG M2 agarose beads (Sigma-Aldrich) and mixed with an equal volume of 2% SDS/25 mM EDTA. At the end, the beads were washed with 40 µl of serum/20 µl of buffer A50 (40 mM Tris-HCl, pH 8, 1 mM EDTA, 10% glycerol, 50 mM NaCl, and 1 mM DTT). Proteins in the beads and supernatant fractions were analyzed by Western blotting to detect RPA and xDNA2.

SSA and end processing assays
These assays were performed essentially as previously described (Yan et al., 2005; Toczylowski and Yan, 2006). The substrate for SSA, pRW4*, was a 5.7-kb linear DNA carrying two 1.2-kb direct repeats at the ends. SSA knockdown inhibits the recruitment of the cell cycle checkpoint kinase ATR to ionizing radiation–induced foci (Zou and Elledge, 2003). ATR is activated by ss-DNA, so this observation is consistent with a role for RPA in stimulating the generation of ss-DNA in addition to its role in directly recruiting ATR through protein–protein interaction with ATR-interacting protein. The insights gained from the Xenopus system should continue to provide detailed understanding of the mechanism responsible for DSB end processing, repair, and checkpoint activation in cells.
2 h, the samples were analyzed by 1% TAE (Tris-acetate-EDTA) agarose gel electrophoresis. Gels were first stained with SYBR gold [Invitrogen] and then dried and exposed to phosphorimag (LAS-2500; Fujifilm).

The RPA protein was purified from cytosol according to a procedure similar to the one previously published (Fang and Newport, 1993). The final concentration of RPA for complementation was ~0.05 μM in the DSB focus experiment and 0.25 μM in the SSA and end processing assays. The T4 gp32 protein [Affymetrix] was used at a 1-μM final concentration in the SSA and end processing experiments.

DNA unwinding assay

The substrate for the unwinding assay was a 48mer double-stranded oligonucleotide with one strand carrying a 5’-biotin and the other strand carrying 21 biotinylated nucleotides on the 3’ half and a 3’-biotin label at the 3’ end (Foczykowski and Yan, 2006). The DNA was coated onto Streptavidin magnetic beads.

The second 48mer (48mer-5) has the sequence of 5’-GGAACACGCTATGACTATGATAC-3’/3’-CTTCTTCTTTTTCTTTTTCCTT-3’ with cold dATP for the second pair), dGTP, TTP, and biotin-dCTP, coated near the 3’ GTGGGTGTTGTGTGGAAC-3’ has the sequence of 5’-CTTTCTTCTTTTTCTTTTTCCTT-3’.

The first 48mer (48mer-1) was annealed with the second 48mer and the mixture was coated onto Streptavidin magnetic beads, and denatured with NaOH according to the procedure described previously (Foczykowski and Yan, 2006). This resulted in two 48mer single-stranded oligonucleotides bound to beads through the biotin-3’ at the 3’. The first 48mer (48mer-1) has the sequence of 5’-GGAACACGCTATGACTATGATAC-3’ and contained two 32P-labeled A (underlined) near the 3’ terminus. The second 48mer (48mer-5) has the sequence of 5’-GGAACACGCTATGACTATGATAC-3’/3’-CTTCTTCTTTTTCTTTTTCCTT-3’.


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