MECHANISTIC ANALYSIS OF A DNA END PROCESSING PATHWAY MEDIATED BY THE XENOPUS WERNER SYNDROME PROTEIN

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The first step of homology-dependent repair of DNA double strand breaks is the strand-specific processing of DNA ends to generate 3' single-strand tails. Despite its importance, the molecular mechanism underlying end processing is poorly understood in eukaryotic cells. We have taken a biochemical approach to investigate DNA end processing in nucleoplasmic extracts derived from the unfertilized eggs of Xenopus laevis. We found that double-strand DNA ends are specifically degraded in the 5' -> 3' direction in this system. The reaction consists of two steps: an ATP-dependent unwinding of double-strand ends and an ATP-independent 5' -> 3' degradation of single-strand tails. We also found that the Xenopus Werner syndrome protein, a member of the RecQ helicase family, plays an important role in DNA end processing. Mechanistically, xWRN is required for the unwinding of DNA ends but not for the degradation of single-strand tails. The xWRN-mediated end processing is remarkably similar to the end processing that has been proposed for the E. coli RecQ helicase and RecJ single-strand nuclease, suggesting that this mechanism might be conserved in prokaryotes and eukaryotes.

DNA double-strand break (DSBs) is the most deleterious type of DNA damage in cells. Improperly repaired DSBs may lead to chromosome truncations or rearrangements and consequently premature cell death or oncogenic cell transformation (1). Currently, three major pathways have been identified to repair DSBs: non-homologous end joining (NHEJ), homologous recombination (HR), and single-strand annealing (SSA) (2-4). HR and SSA share some mechanistic features such as dependence on sequence homology and strand-specific end processing. Moreover, mitotic recombination appears to proceed via synthesis-dependent strand annealing (SDSA), which is essentially a hybrid mechanism including features of both classical HR and SSA (5-7).

The first step of homology-based repair is the processing of DSBs into 3' single-stranded tails. In Escherichia coli, the RecBCD helicase/nuclease complex constitutes the major pathway for end processing. In addition, the RecQ helicase and the RecJ ss-DNA nucleases have been proposed to constitute an alternative pathway (8). In eukaryotes, however, the mechanism for end processing is poorly understood. Genetic analysis in the yeast Saccharomyces cerevisiae has suggested that the Mre11-Rad50-Xrs2 (MRX) complex plays an important role in end processing, but exactly what it does in this reaction remains obscure (9). Mre11 is endowed with both an exonuclease and an endonuclease activity (10-13). However, the directionality of the Mre11 exonuclease activity is 3' -> 5', which is opposite of what is expected of an exonuclease that resects DNA in the 5' -> 3' direction. Moreover, a mutation that knocks out the nuclease activity without disrupting MRX complex assembly shows no significant defect in the processing of HO-induced DSBs in mitotic cells, whereas other nuclease-inactivating mutations that block end processing appear to do so indirectly by destabilizing the MRX complex (14-16). Another candidate nuclease is ExoI, which, when over-expressed, can suppress the mitotic DNA repair defect of mre11, rad50, and xrs2 mutants (17). However, an exoI null mutant has no significant defect in recombinational repair (18,19) and only minor defect in DNA end processing (15).

While genetic analysis has provided many fundamental insights into DSB repair, some important mechanistic questions, including DNA end processing, can be addressed more conveniently and rigorously in a biochemical system. One powerful biochemical system is the extracts derived from the interphase eggs of the frog Xenopus laevis. This system can efficiently
join various DNA ends via a Ku-dependent NHEJ mechanism (20,21). It also contains robust activity for SSA repair of homologous ends, which was demonstrated first in oocytes and later in nucleoplasmic extract (NPE) derived from nuclei reconstituted in *Xenopus* egg extracts (22,23). The NPE system has made it possible to use the powerful immunodepletion procedure to analyze the function of various proteins in SSA. One of the major findings from this analysis is that the *Xenopus* Werner syndrome protein (xWRN) plays an important role in SSA (23). The WRN protein, a member of the RecQ helicase family, has been implicated in various aspects of genome maintenance, such as DNA replication, NHEJ, homology-based DSB repair, and telomere maintenance (24,25). However, the exact mechanistic role of WRN in such diverse DNA transactions remains a mystery.

To better understand homology-dependent DSB repair and the role of xWRN in it, we have initiated a biochemical analysis of DNA end processing using NPE as the model system. Previously, it has been observed that linear DNA injected into *Xenopus* oocytes is degraded in a 5'->3' strand-specific manner (26). However, the reaction mechanism and the proteins participating in end processing were not pursued. We have found that NPE also contains robust activity for 5' strand-specific processing of double-strand DNA ends. This reaction is dependent on ATP and can be separated into two steps: the unwinding of DNA ends and the degradation of the 5' ss-tail. The unwinding step, but not the single-strand DNA degradation step, is dependent on ATP. Moreover, we have found that the xWRN helicase plays an important role in DNA end processing by promoting end unwinding. These results provide the first biochemical evidence that DNA end processing in eukaryotes is a coupled end unwinding/5' ss-tail degradation reaction and that WRN, a member of RecQ helicase family, acts as the primary helicase for unwinding of ds-DNA ends in NPE. Interestingly, the *E. coli* RecQ helicase and RecJ ss-DNA exonuclease have been proposed to act in a similar way to process DNA ends, suggesting that a conserved DNA end processing pathway might be present in prokaryotes and eukaryotes.

**EXPERIMENTAL PROCEDURES**

*Extract preparation -* Crude interphase *Xenopus* egg extracts and demembranated sperm chromatin were prepared following the published procedures (27). Nucleoplasmic extracts (NPE) were prepared from nuclei reconstituted around sperm chromatin in crude extracts according to the protocol described by Walter and Newport (28).

*DNA substrate preparation -* The following five oligonucleotides were used to prepare the various oligonucleotide-based DNA substrates in this study. 24mer 1: 5' GGAAACAGCTATGACCATTAC 3'; 48mer: 5' GTTCACACAAACACCCACCAACCGTAAT CATGGTCATAGCTTCCC 3'; biotin-48mer: same as 48mer except that the 5' G is biotinylated; 24mer 2: 5' TAAACAGCTATGACCATTACGT 3'; 47mer: 5' Biotin-GACCAACCACACACACCAACACACCAT GC CATGGTCATAGCTTCCC 3'; biotin-48mer: same as 48mer except that the 5' G is biotinylated; 24mer 2 and 47mer were annealed and extended with 32P dATP, dCTP, dGTP, and TTP by Klenow (exo-; NEB). To make the 3' oligo duplex (3' accessible), 24mer 2 and 47mer were annealed and extended with 32P-dATP, dCTP, dGTP, and TTP by Klenow. The nuclease-resistant thio 5' oligo duplex was prepared with 24mer 1 and biotin-48mer and the fill-in reaction was performed in the presence of thio-dGTP, thio-TTP, 32P dATP, and dCTP. Long linear DNA was made by digesting pUC19 with *BamHI* and then filling the ends with dGTP, 32P dATP, and ddTTP. Single-stranded DNA was prepared by denaturing DNA by heat. Coating of biotin-containing oligos on Streptavidin paramagnetic beads (Dynal) was carried out following the manufacturer’s instruction.

*DNA end processing assays -* A typical end processing assay contains: 4µl NPE, 0.8µl 10xATP mix (20mM ATP, 200mM phosphocreatine, and 0.5mg/ml creative kinase), 1µl DNA (approximately 40ng/µl for pUC19 or 0.2ng/µl for oligo DNA), and 4.8µl ELB buffer (10mM HEPES (pH7.5), 250mM sucrose, 2.5mM MgCl2, 50mM KCl, and 1mM DTT). Reactions with depleted NPE usually contain: 5µl depleted NPE, 0.6µl 10x ATP mix, 0.75µl DNA, 1µl ELB buffer or xWRN protein. The reactions were
incubated at room temperature, and samples were taken at the indicated times. For reactions that required separation of beads and supernatants, samples were mixed with 4 volumes of washing buffer (10mM Tris-HCl (pH8), 1mM EDTA, 1M NaCl, and 0.05% NP40) and beads were separated by magnet. The supernatants were withdrawn and the beads were washed once with washing buffer. All samples were finally mixed with equal volume of 2x sample buffer (80mM Tris-HCl (pH8), 0.13% phosphoric acid, 8mM EDTA, 5% SDS, 0.2% bromophenol blue, and 10% Ficoll). The volume was brought up to 10μl with 1x sample buffer and then 1μl proteinase K (10mg/ml) was added. After overnight incubation at room temperature, samples were separated on either 1% TAE/agarose gel, or 8% TAE/polyacrylamide gel, or 12% urea/polyacrylamide gel. Gels were dried and exposed to Phosphorimager (Fuji) and X-ray film.

**Immunodepletion** - Immunodepletion of xWRN from NPE has been described previously (23). For the complementation experiments, xWRN was purified directly from cytosol as previously described (23,29) and *E. coli* RecQ protein was obtained from Dr. Stephen C. Kowalczykowski. The final concentrations of xWRN in the complementation reactions were 5ng/μl (approximately 30-40% of the estimated endogenous xWRN concentration) and 10ng/μl (approximately equivalent molar concentration to that of the endogenous xWRN).

**RESULTS**

**NPE can efficiently process double-strand DNA ends in the 5' -> 3' direction** - To study ds-DNA end processing and the strand specificity of processing, we prepared two types of oligonucleotide duplexes that carry a biotin moiety at either the 5' end or the 3' end. After binding to streptavidin-paramagnetic beads, the biotin end is blocked, leaving only the opposite end accessible to the end processing machinery. For simplicity, these oligo duplexes will be referred to as: 5’ duplex (biotin at 3’ end; 5’ end accessible; Fig. 1A and B) and 3’ duplex (biotin at 5’ end; 3’ end accessible; Fig. 1B). In addition, the oligos were labeled with 32P at either the 3’ end (for 3’ duplex) or immediately inside the 3’ end (for 5’ duplex).

We first determined the fate of the 5’ duplex in NPE. The DNA was coated on beads and then incubated in NPE. The beads were isolated at various times, heat denatured, and separated by urea-polyacrylamide gel electrophoresis (urea PAGE). As shown in Fig. 1A, the labeled DNA recovered from the beads was degraded over time. The reaction was rapid, and the intermediates were most clearly observed at the 3 minute time point. The final products were a mixture of nucleotides estimated to be 4-11 nucleotides in size. The incomplete degradation is mostly likely due to the physical hindrance to the nuclease(s) as it approached the beads.

The pattern of degradation observed above is consistent with a 5' -> 3' end processing. However, an endonucleolytic reaction might also generate a similar pattern. In addition, because the 3' end was linked to the beads, any 3' -> 5' degradation activity would have escaped detection. To distinguish among these possibilities, we next determined the fate of the 3' duplex in NPE. The beads pre-coated with either the 5’ duplex (as control) or the 3’ duplex were incubated in NPE for various times. A 3’ -> 5’ exonuclease or an endonuclease would immediately release the 32P label from the 3’ duplex into the supernatant. We therefore analyzed the whole reaction instead of just the beads. The samples were directly treated with SDS-containing sample buffer and proteinase K and then separated by native polyacrylamide gel electrophoresis (native PAGE). (Native PAGE was used because NPE contains materials that interfere with urea PAGE). The condition used here cannot disrupt the interaction between biotin and streptavidin (disruption requires harsh conditions such as boiling in SDS or 65°C-90°C incubation in 95% formamide (Dynal)), so the DNA bound to beads cannot enter the gel unless first being denatured by heat. As shown in Fig. 1B, the 3’ duplex was stable, with the 32P label largely retained on the beads even after 30 minutes of incubation. Upon heat treatment, the labeled DNA was released from the beads, but migrated at the position of intact size, suggesting that it was not subject to any significant degradation. The 5’ duplex behaved as expected from Fig. 1A. The 32P label was also largely retained on the beads, but upon heat treatment, the released labeled DNA was mostly of small size (4-11 nucleotides). (The small amount of dAMP generated was later...
converted to dATP, presumably by adenylate kinase and nucleoside diphosphate kinase in NPE.) Taken together, these results strongly suggest that ds-DNA end processing in NPE is strand specific and proceeds mostly in the 5' -> 3' direction.

End processing in NPE is highly processive -
In yeast, end processing can proceed over an extensive length, reaching one kilo-bases or more (15,30,31). In NPE, SSA can occur between two 1.2kb repeats, suggesting that DNA up to this length could be degraded from the 5' strand. To directly test the processivity of end processing in NPE, we prepared a 2.6kb linear DNA (pUC19 linearized by BamHI) as substrate. Such long linear DNA can be rapidly repaired in NPE by NHEJ, a situation that complicates the detection of end processing. To overcome this problem, we filled in the 3' recessed end (GATC) with dGTP, 32P-dATP, and ddTTP. The resulting DNA cannot be repaired by NHEJ due to the block by ddTTP. When this DNA was incubated in NPE, it was gradually degraded over time as judged by SYBR Gold staining (Fig. 1C, left panel). Notably, the partially degraded intermediates still largely retained the 3' end 32P label (Fig. 1C, middle panel). For example, as shown in Fig. 1C, right panel, after 7 minutes, most of the DNA was at least partially degraded based on SYBR Gold staining, yet the total amount of 32P label on these DNA molecules was not significantly reduced. These data suggest that NPE can also process long linear DNA molecules in the 5' -> 3' direction over a length of up to 2.6 kilo-bases.

Processing of ds-DNA ends is dependent on ATP -
To understand the mechanism underlying 5' -> 3' ds-DNA end processing, we determined if the reaction depends on ATP, a high energy molecule required for many biochemical reactions. NPE normally contains a significant amount of ATP. This endogenous ATP was removed by treatment with apyrase, an enzyme that is often used to destroy ATP in Xenopus egg extracts (32). The DNA substrates, 5' oligo duplex and linear pUC19 DNA, were then incubated in the ATP-depleted NPE or control NPE. Samples were taken at various times, treated with SDS and proteinase K, and separated by native PAGE (for oligo duplex) or on an agarose gel (for pUC19). As shown in Fig. 2, in the apyrase-treated NPE (-ATP), both DNA substrates were stable. In contrast, in the control NPE (+ATP), the two DNAs were rapidly degraded into smaller products. These results indicate that ds-DNA end processing in NPE is strictly dependent on ATP.

Processing of ds-DNA ends is coupled to DNA unwinding -
One likely mechanistic explanation for the ATP dependence is that ds-DNA end processing might require DNA unwinding, a reaction that depends on ATP hydrolysis for energy. However, the type of DNA substrates used in the above experiments does not permit the detection of DNA unwinding because the unwound DNA is also degraded. To overcome this problem, we made a nuclease-resistant oligo duplex by using thio-dGTP and thio-TTP in the fill-in reaction of a 24mer/48mer partial duplex. Thionucleotides confer resistance to degradation by many nucleases, including the nuclease(s) responsible for end processing in NPE (Fig. S1). The 48mer carries a biotin moiety at the 5' end, while the thio strand carries two 32P label near the 3' end. After binding to Streptavidin beads, the thio strand will carry an accessible 5' end and a 32P label near the 3' end (and beads). For simplicity, we will refer to this DNA as thio 5' duplex (Fig. 3A). After incubation in NPE, if there is no unwinding, the thio strand should remain annealed to the complementary biotin-carrying strand and thus bound to the beads. Conversely, if there is unwinding, the thio strand should be released from the beads into the supernatant.

The thio 5' duplex was incubated in NPE for various times, the beads and the supernatants were separated from each other, and the DNA in each fraction were analyzed by native PAGE. As shown in Fig. 3B, the partially degraded, thio-containing DNA was recovered exclusively in the supernatant, whereas the residual DNA on the beads were all of intact size (when analyzed after being released from beads by heat). Importantly, the release of the thio strand was strictly dependent on ATP. As shown in Fig. 3C, when ATP was removed from NPE, the thio strand remained bound to the beads. These results strongly suggest that ds-DNA end processing is tightly coupled to an ATP-dependent DNA unwinding reaction.
Ss-DNA ends are efficiently processed in the 5’ -> 3’ direction - If unwinding is an integral step of ds-DNA end processing, one prediction would be that ss-DNA ends are the target for nucleolytic degradation. Moreover, this degradation would have to be mostly 5’ -> 3’ to ensure that, of the two tails (5’ and 3’) generated by unwinding, only the 3’ ss-tail remains as the final product. To test this prediction, we denatured the 5’ oligo duplex and the 3’ oligo duplex and then coated the biotin-carrying ss-oligos onto Streptavidin magnetic beads. One oligo had a free 5’ end but a blocked 3’ end (5’ ss-oligo). The other oligo had a free 3’ end but a blocked 5’ end (3’ ss-oligo). Both ss-oligos also carried a 32P label at or near the 3’ end. These bead-bound ss-oligos were then incubated in NPE. Samples were taken at various times, treated with SDS/proteinase K, heated, and separated by native PAGE. As shown in Fig. 4A, the oligo with the free 5’ end was rapidly degraded. In contrast, the oligo with the free 3’ end was quite stable, with only a small amount of degradation after extensive incubation. (As will be shown later, long linear ss-pUC19 DNA can also be efficiently degraded in NPE). Notably, 5’ -> 3’ ss-DNA end processing, unlike ds-DNA end unwinding, was equally efficient in NPE pre-treated with apyrase (-ATP) or buffer (+ATP) (Fig. 4B). Together, these data indicate that NPE can efficiently degrade ss-DNA ends in an ATP-independent reaction and does so almost exclusively in the 5’ -> 3’ direction. They also suggest, when combined with the unwinding data above, that ds-DNA end processing can be dissected into two distinct steps based on ATP dependence. The unwinding step is absolutely dependent on ATP, but once this is complete, ATP is no longer required for the degradation of 5’ ss-DNA tails.

XWRN is important for ds-DNA end processing - We next attempted to identify the proteins that participate in ds-DNA end processing. Previously we had shown that the Xenopus Werner syndrome protein (xWRN) is important for SSA repair which is dependent on end processing (23). We therefore determined if xWRN might be important for ds-DNA end processing. To do this, xWRN was depleted from NPE with affinity-purified antibodies to at least 98% efficiency (for details, see (23)). The linear pUC19 DNA as that used in Fig. 1C was incubated in either xWRN-depleted or mock-depleted NPE. In mock-depleted NPE, pUC19 was efficiently degraded as judged by both total DNA staining and 32P label at the 3’ end (Fig. 5A). (The reaction had a slower kinetics because the depletion procedure reduced the overall potency of NPE due to unavoidable dilution and extensive incubation). In xWRN-depleted NPE, however, pUC19 was very stable, even after 120 minutes of incubation. Importantly, this deficiency could be complemented by the addition of the purified xWRN protein to the xWRN-depleted NPE (Fig. 5B). The xWRN protein used in this experiment was purified by multiple steps of chromatography without involving anti-xWRN antibodies (23,33). By itself xWRN did not cause any detectable degradation to pUC19 (Fig. 5B), indicating that the protein was free of contaminating nucleases that might have inadvertently degraded DNA in a non-specific way. In addition, a different helicase, the E. coli RecQ protein (34), was unable to complement the defect (Fig. 5C). Collectively, these data strongly suggest that xWRN is a critical player in the processing of ds-DNA ends.

XWRN acts in end unwinding but not in ss-DNA end degradation - As shown earlier, the processing of ds-DNA ends can be divided into two steps: the unwinding of ends and the degradation of 5’ ss-tails. An important mechanistic question is which step requires xWRN. To evaluate xWRN’s role in ss-DNA degradation, we prepared linear ss-DNA by heat-denaturing the 3’-labeled ds-pUC19 DNA. The denatured ss-DNA was then incubated in xWRN- or mock-depleted NPE and then analyzed by agarose gel electrophoresis. As shown in Fig. 6A, the ss-pUC19 DNA was rapidly degraded in both reactions as judged by both total DNA staining and 32P signal. (The multiple slower-migrating faint bands in the control reaction that contains buffer only are pUC19 ss-DNA molecules with different conformations). This suggests that xWRN does not play any significant role in the degradation of ss-tail DNA. To examine the role of xWRN in DNA unwinding, we incubated the thio 5’ duplex in either xWRN- or mock-depleted NPE. Samples were taken at various times and the beads and the supernatants were then separated. In mock-depleted NPE, as expected, a significant fraction of the thio strand was released from the
beads into the supernatant (Fig. 6B). In xWRN-depleted NPE, however, most of the labeled strand remained bound to the beads and failed to enter the gel. This defect was specific and could be complemented by the addition of the purified xWRN protein to the xWRN-depleted NPE (Fig. 6C). Similar to the result with end processing, RecQ was unable to complement the unwinding defect (Fig. 6D). Together, these results strongly suggest that xWRN participates in ds-DNA end processing by promoting the unwinding of DNA ends with no significant effect on the degradation of the resulting ss-DNA tails.

DISCUSSION

The major observations of this study are: (1) NPE contains robust activity for 5' -> 3' processing of ds-DNA ends; (2) the reaction can be separated into two steps: the unwinding of DNA ends and the degradation of 5' ss-DNA tails; (3) the unwinding step is dependent on ATP, but the ss-DNA end processing is not; (4) xWRN is important for ds-DNA end processing; and (5) xWRN acts at the unwinding step but not the ss-DNA degradation step. Importantly, xWRN is also required for SSA (23). This strongly suggests that the 5’ strand-specific processing of DNA ends in NPE is not merely a DNA degradation reaction. Instead, the resulting 3’ strand can be efficiently channeled into a homology-dependent DSB repair pathway.

These observations suggest the following mechanism for ds-DNA end processing in NPE (Fig. 7A). The first step is that xWRN unwinds ds-DNA ends into ss-DNA tails in an ATP dependent reaction. Although WRN has limited 3' -> 5' exonuclease activity, it is of the wrong polarity and inactive on ss-DNA (35,36) and as such highly unlikely to be directly responsible for the degradation of the 5’ strand. Instead, our data suggest that an ATP-independent exonuclease degrades specifically the 5’ ss-tail. The lack of a significant 3’ – 5’ ss-DNA exonuclease activity ensures that the 3’ ss-tail remains as the final product. Importantly, blocking end unwinding, by depleting either xWRN or ATP, also blocks end processing. End unwinding is thus an obligatory first step for end processing.

The basic architecture of this pathway is remarkably similar to that of the RecQ/RecJ model of E. coli (Fig. 7B). There the RecQ helicase has been proposed to unwind DNA ends and then the RecJ ss-DNA exonuclease degrades the 5’ ss-tail, leaving behind the 3’ ss-tail. In the Xenopus system, xWRN, which is also a member of the RecQ helicase family, appears to play a similar role as that of the E. coli RecQ. The 5' -> 3' ss-DNA exonuclease in NPE has yet to be identified. Although xWRN has an exonuclease domain at its N terminus, the polarity is 3’ -> 5’ instead of 5’ -> 3’ and our data also showed that xWRN is not the nuclease. ExoI is a good candidate, but further studies are required to reveal the true identity of the nuclease (18). Clearly, a RecQ helicase and an 5’ -> 3’ ss-exonuclease are only the basic mechanical components for end processing. Other proteins, such as MRX, CDK1, and ATM could play a regulatory role in end processing (37,38).

Can this mechanism be applied to other eukaryotes? The role of WRN in end processing has not been studied so far, but this protein is recruited to DSBs and plays an important role in homology-dependent DSB repair in both human cells and Xenopus egg extracts (23,39,40). Although none of the RecQ helicases in other organisms is essential for homologous recombination and, by inference, for ds-DNA end processing, the coupled end unwinding/ss-DNA degradation might be carried out by multiple combinations of helicases and ss-DNA nucleases. Indeed, the helicases and the nucleases of the two E. coli end processing pathways, RecBCD and RecQ/RecJ, are known to be able to act in various combinations (41). In higher eukaryotes, there are multiple RecQ helicases. Although the E. coli RecQ protein is unable to substitute for xWRN in end processing, this is most likely due to the lack of protein-protein interaction with other components of the end processing machinery. Other RecQ helicases, such as the Bloom syndrome protein (BLM), might also be able to unwind DNA from ends. In S. cerevisiae, Sgs1 is known to be functionally redundant with other helicases such as Dna2 (42). In NPE, xWRN appears to be the major RecQ helicase, probably due to the developmentally regulated expression of various RecQ helicases (data not shown). In other organisms, therefore, inactivating a single RecQ helicase might not have a dramatic effect on end processing. However, the basic mechanism of unwinding/ss-DNA degradation is very likely to
be operative in these organisms by the combined action of DNA helicases and ss-DNA exonucleases.

The finding that xWRN participates in ds-DNA end processing might provide a unified mechanistic explanation for WRN's role in genome maintenance. Loss of WRN affects many aspects of DNA transactions, including homology-directed DSB repair, replication restart, and telomere maintenance. Strand-specific processing of DSB ends is a pre-requisite for not only SSA but also HR, WRN might therefore play a similar role in HR. This might be particularly important for the telomerase-independent maintenance of telomeres, which is apparently achieved by a HR-like mechanism (43). As to replication fork restart in general, the results from this study imply that WRN might be recruited to dissociate the stalled newly synthesized strand for degradation. Indeed, genetic analysis in E. coli has suggested that RecQ and RecJ might act in this way to expand the gap between a fork-stalling lesion and the next Okazaki fragment (44). Future studies analyzing WRN's role in unwinding DNA ends or stalled forks for degradation should help reveal the mechanistic role of this important protein in genome maintenance.

THE ABBREVIATIONS USED ARE: DNA double-strand break: DSB; Werner syndrome protein: WRN

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Figure legends

**Fig. 1.** 5’ -> 3’ strand-specific processing of ds-DNA ends in NPE. (A) Processing of the 5’ oligo duplex bound onto Streptavidin magnetic beads. After incubation in NPE for the indicated times, the beads were isolated and the DNA retained on the beads was denatured and separated by urea PAGE. The final products range from 4-11 nucleotides in size. (B) Comparison of the processing of the 3’ oligo duplex and the 5’ oligo duplex. The two oligos were coated onto beads and then incubated in NPE for the indicated times. Samples were directly treated with sample buffer, split into two aliquots, one of which denatured by heat, and separated by native PAGE. The products from the 5’ oligo duplex ran together due to the lower resolution of native PAGE. (C) 5’ -> 3’ strand-specific processing of pUC19. Linear pUC19 with a ddTTP at the 3’ end and a 32P label immediately inside was incubated in NPE for the indicated times and separated on an agarose gel. The gel was stained with SYBR Gold for detection of DNA (left panel) and then dried for detection of the 32P signal by Phosphoimager (middle panel: image; right panel: plot of the 32P signal in each lane (after subtraction of background) against time).

**Fig. 2.** ATP dependence of ds-DNA end processing. NPE was treated with either apyrase (20ng/µl; -ATP) or buffer (+ATP) at room temperature for 15min and then used for end processing assays. (A) End processing of the 5’ oligo duplex. Samples were separated on by native PAGE and the 32P signal was detected by Phosphoimager. (B) End processing of linear pUC19. Samples were separated on an agarose gel and the 32P signal was detected by Phosphoimager.

**Fig. 3.** DNA unwinding during ds-DNA end processing. (A) The the substrate, thio 5’ duplex, for the unwinding assay. (B) Unwinding of the thio 5’ duplex. The DNA pre-coated onto Streptavidin magnetic beads was incubated in NPE. Samples were taken at the indicated times and separated into bead and supernatant fractions. Each fraction was split into two aliquots, one of which then denatured by heat. The products were separated by native PAGE and the 32P signal was detected by Phosphoimager. (C) Effect of ATP on unwinding. Similar to (B) except that NPE was pre-treated with apyrase (20ng/µl; -ATP) or buffer (+ATP) at room temperature for 15min.

**Fig. 4.** 5’ -> 3’ processing of ss-DNA ends. (A) Preferential processing of the 5’ end of ss-DNA. The 5’ oligo duplex and the 3’ oligo duplex were denatured by heat and then coated onto Streptavidin magnetic beads. The bead-coated ss-DNA (5’ ss-oligo and 3’ ss-oligo) was incubated in NPE. Samples were taken at the indicated times, denatured by heat, and separated by native PAGE. (B) ATP dependence of ss-DNA end processing. The 5’ ss-oligo on beads was incubated in NPE that had been pre-treated with apyrase (-ATP) or buffer (+ATP). Samples were analyzed in the same way as in (A).

**Fig. 5.** Effect of xWRN on the processing of ds-DNA ends. (A) Linear pUC19 DNA was incubated in xWRN-depleted or mock-depleted NPE. Samples were treated with sample buffer and separated on an agarose gel. The gel was stained with SYBR Gold and then dried for exposure to Phosphoimager. Top: SYBR Gold staining. Bottom: 32P signal. (B) Rescue of end processing by the purified xWRN protein. Linear pUC19 DNA was incubated in xWRN-depleted NPE supplemented with the purified xWRN protein or buffer. Two additional reactions, pUC19 incubated with xWRN or buffer, served as controls. The samples were analyzed as in (A). (C) Rescue of end processing by the purified *E. coli* RecQ protein. Linear pUC19 DNA was incubated in xWRN-depleted NPE supplemented with the purified RecQ (10ng/µl) or buffer.

**Fig. 6.** Mechanistic role of WRN in ds-DNA end processing. (A) Effect of xWRN on ss-DNA end processing. Denatured pUC19 DNA was incubated in xWRN-depleted or mock-depleted NPE. Samples were separated on a TAE agarose gel, which was stained with SYBR Gold and then dried for exposure to Phosphoimager. The fast-migrating band detected by SYBR Gold stain was the dye front. (B) Effect of xWRN on DNA unwinding. The thio 5’ oligo duplex pre-coated onto beads was incubated in xWRN-
depleted or mock-depleted NPE. At the indicated times, samples were withdrawn, separated into bead and supernatant fractions, and analyzed by native PAGE. B: beads; S: supernatant. (C) Rescue of unwinding by the purified xWRN protein. The thio 5’ oligo duplex pre-coated onto beads was incubated in xWRN-depleted NPE supplemented with the purified xWRN protein or buffer. Samples were analyzed in the same way as in (B). (D) Rescue of unwinding by *E. coli* RecQ (10ng/μl). The thio 5’ oligo duplex pre-coated onto beads was incubated in xWRN-depleted NPE supplemented with the purified RecQ or buffer.

**Fig. 7.** Model of ds-DNA end processing. (A) End processing in NPE. xWRN unwinds ds-DNA ends and the resulting 5’ ss tail is degraded by an yet to be identified 5’ -> 3’ exonuclease. The final product is a 3’ ss tail. Other proteins that might modulate the activity of these proteins are not depicted. (B) A model for the RecQ mediated end processing in *E. coli*. RecQ helicase unwinds ds-DNA ends and the resulting 5’ ss tail is degraded by RecJ.
Fig. 1
Fig. 2

A

5' 3' 5' 3' 5' 3' 5' 3' 5' 3' 5'

+ Apyrase (-ATP)  + Buffer (+ATP)

0' 3' 10' 30' 3'10' 30' 3'10' 30' 3'10' 30' 3'10' 30'

B

5' Linear pUC19 3' 5' 3' 5'

+ Buffer (+ATP)  + Apyrase (-ATP)

0' 5' 10' 15' 20' 25' 30' 35' 40' 45' 50' 55' 60' 65' 70' 75' 80' 85' 90' 95' 100'
Fig. 5
Fig. 6
Fig. 7
Mechanistic analysis of a DNA end processing pathway mediated by the xenopus werner syndrome protein
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