II. CHARACTERIZATION OF THE INTEGRAL 3′ → 5′ DNA EXONUCLEASE*

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In addition to its DNA helicase activity, Werner syndrome protein (WRN) also possesses an exonuclease activity (Shen, J.-C., Gray, M. D., Kamath-Loeb, A. S., Fry, M., Oshima, J., and Loeb, L. A. (1998) J. Biol. Chem. 273, 34139–34144). Here we describe the properties of nearly homogeneous WRN exonuclease. WRN exonuclease hydrolyzes a recessed strand in a partial DNA duplex but does not significantly digest single-stranded DNA, blunt-ended duplex, or a protruding strand of a partial duplex. Although DNA is hydrolyzed in the absence of nucleoside triphosphates, nuclease activity is markedly enhanced by a 3′-OH group. WRN exonuclease is distinguished from known mammalian DNA nucleases by its covalent association with a DNA helicase, preference for a recessed DNA strand, stimulation by ATP, ability to equally digest DNA with 3′-OH or 3′-PO4 termini, and its preference for a DNA strand terminating with two mismatched bases. WRN exonuclease is distinguished from known mammalian DNA nucleases by its covalent association with a DNA helicase, preference for a recessed DNA strand, stimulation by ATP, ability to equally digest DNA with 3′-OH or 3′-PO4 termini, and its preferential digestion of DNA with a single 3′-terminal mismatch.

Werner Syndrome (WS)1 is a recessive inherited disease characterized by genetic instability and aging in early adulthood (1, 2). The gene defective in WS, WRN, encodes a 3′ → 5′ RecQ-like DNA helicase that unwinds DNA in an ATP-dependent manner (3–5). Mutations in WRN are invariably found in patients exhibiting the clinical symptoms of WS (6, 7). These include atherosclerosis, osteoporosis, diabetes mellitus, and bilateral cataracts, as well as an unusually high incidence of tumors of non-epithelial cell origin. At the cellular level, WS cells are characterized by chromosomal translocations, large DNA deletions, elevated rates of homologous recombination, and defective maintenance of telomeres, and a prolonged S-phase of DNA synthesis (8–15).

In the preceding paper (16), we reported the identification of a novel exonuclease activity in WRN. We used molecular genetic, biochemical, and immunochemical methods to establish that the exonuclease, like the DNA helicase, is integral to WRN. Although the two activities are expressed in the same polypeptide in the wild-type protein, they can be uncoupled from each other by introducing mutations separately in each of the two domains. In patients, mutations in WRN are not necessarily located in the helicase domain. They are found throughout the gene and invariably introduce stop codons or deletions (6, 7). It has been argued that many mutations obliterate the nucleotide localization signal and that lack of localization may be important in the pathogenesis of WS (17, 18). This lack of nuclear localization would result in deficits of both helicase and exonuclease activities.

To gain a better understanding of the functions of the WRN exonuclease, we have studied its properties in some detail. We report the following characteristic features of the WRN exonuclease: 1) it hydrolyzes DNA in a 3′ → 5′ direction in an ATP-stimulated reaction to generate 5′-deoxyribonucleoside monophosphate products, 2) its preferred substrate is a recessed strand of a partial DNA duplex that terminates with either a 3′-OH or 3′-PO4 group, and 3) it efficiently digests DNA with a single 3′-terminal mismatched nucleotide but does not degrade DNA with two 3′-terminal mismatches, nor does it hydrolyze single-stranded DNA.

EXPERIMENTAL PROCEDURES

Materials and Enzymes—[γ-32P]ATP and [α-32P]dCTP were products of NEN Life Science Products. Bacteriophage T4 polynucleotide kinase and Klenow fragment of E. coli DNA polymerase I were supplied by New England Biolabs. Deoxyribonucleoside triphosphates (dNTPs) were purchased from Perkin-Elmer. Ribonucleoside triphosphates (NTPs) were supplied by Amersham Pharmacia Biotech. High performance liquid chromatography purified oligodeoxyribonucleotides listed in Table I were provided by Operon Technologies. Dithiothreitol (DTT), Nonidet P-40, AMP, cyclic AMP, and cyclic GMP were purchased from Sigma. DEAE (DE81) and Whatman No. 3MM filter paper were provided by Whatman. Polyethyleneimine-Cellulose F thin layer chromatography plates were purchased from VWR.

DNA Labeling and Annealing—Single-stranded DNA oligomers were labeled by 32P at their 5′-end and annealed to complementary unlabeled DNA oligomers as described in the preceding paper (16). To label the 20-mer DNA at its 3′-end, it was annealed to the 46-mer oligonucleotide (Table I), and its 3′-terminus was extended by a single complementary 5′-[32P]dCMP residue in a reaction catalyzed by the Klenow fragment of E. coli DNA polymerase I. The reaction mixture contained in a final volume of 10 μl, 25 mm Tris-HCl buffer, pH 8.0, 10 mM MgCl2, 40 mM KCl, 5 μM dCTP, 5 μM of 5′-[32P]dCTP, 10 μg of bovine serum albumin, 16 pmol of 20-mer/46-mer hybrid DNA, and 0.5 unit of Klenow DNA polymerase. Following incubation for 10 min at 37 °C, incorporation of 5′-[32P]dCMP was terminated by the addition of denaturing loading buffer; the samples were boiled, and DNA was electrophoresed through a 14% polyacrylamide-urea gel (19). The resolved 32P-labeled 3′-21-mer oligomer was isolated using a Bio-Rad model 160 staining gel apparatus and visualized by autoradiography.

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1 The abbreviations used are: WS, Werner syndrome; DTT, dithiothreitol; ATP-γ-S, adenosine 5′-O-(thiotriphosphate); 4NQO, 4-nitroquinoline 1-oxide.
WRN exonuclease selectively hydrolyzes the recessed strand in a partial DNA duplex. WRN protein (15 fmol) was incubated with a mixture of 0.03 pmol of each 5'-32P-labeled DNA substrate and increasing amounts of the same unlabeled DNA molecule at 37 °C for 10 min. Control reactions containing each of the 32P-labeled DNA substrates were incubated in the absence of WRN. Following termination of nuclelease action, products of DNA digestion were resolved by electrophoresis through a 14% polyacrylamide-urea gel. The schematically drawn DNA substrates are (left to right): partial duplex of 32P-labeled 5'-20 mer and unlabeled 46 mer; blunt-ended duplex of 32P-labeled 5'-20 mer and unlabeled anti-20 mer; partial duplex of 32P-labeled 5'-46 mer and unlabeled 20 mer; single-stranded 32P-labeled DNA substrate and increasing amounts of the same unlabeled DNA at 37 °C for 10 min. Control reactions containing each of the 32P-labeled DNA substrates were incubated in the absence of WRN. Following termination of nuclease action, products of DNA digestion were resolved by electrophoresis through a 14% polyacrylamide-urea gel. The schematically drawn DNA substrates are (left to right): partial duplex of 32P-labeled 5'-20 mer and unlabeled 46 mer; blunt-ended duplex of 32P-labeled 5'-20 mer and unlabeled anti-20 mer; partial duplex of 32P-labeled 5'-46 mer and unlabeled 20 mer; single-stranded 32P-labeled 5'-20 mer.

**FIG. 1.** WRN exonuclease selectively hydrolyzes the recessed strand in a partial DNA duplex. WRN protein (15 fmol) was incubated with a mixture of 0.03 pmol of each 5'-32P-labeled DNA substrate and increasing amounts of the same unlabeled DNA molecule at 37 °C for 10 min. Control reactions containing each of the 32P-labeled DNA substrates were incubated in the absence of WRN. Following termination of nuclelease action, products of DNA digestion were resolved by electrophoresis through a 14% polyacrylamide-urea gel. The schematically drawn DNA substrates are (left to right): partial duplex of 32P-labeled 5'-20 mer and unlabeled 46 mer; blunt-ended duplex of 32P-labeled 5'-20 mer and unlabeled anti-20 mer; partial duplex of 32P-labeled 5'-46 mer and unlabeled 20 mer; single-stranded 32P-labeled 5'-20 mer.

**RESULTS**

WRN Exonuclease Preferentially Hydrolyzes a Recessed Strand in a Partial DNA Duplex—To determine the DNA substrate requirements of WRN nuclease, we examined the ability of WRN to hydrolyze single-stranded DNA, blunt-ended DNA duplex, and a partial duplex in which one of the DNA strands has 3' and 5' protruding tails relative to the other. A fixed amount of each of the 32P-labeled DNA substrates was mixed with increasing amounts of the same unlabeled DNA and incubated with WRN as described. Measurements of the exonuclease activity of WRN demonstrated that only a recessed strand in a partial DNA duplex was significantly hydrolyzed by the enzyme (Fig. 1). Quantitation of the amount of DNA digested as a function of substrate concentration yielded a $K_m$...
value of $22 \times 10^{-9}$ M. No detectable WRN nuclease activity was observed with the other DNA substrates even when they were used at 100-fold higher concentrations. This suggests that the lack of exonucleolytic activity with these substrates is not simply reflective of lower binding affinities. Furthermore, WRN helicase was able to unwind the partial duplex irrespective of the strand that was 5'-end-labeled (data not shown). This again indicates that, at least with the 3' -protruding DNA substrate, a lower affinity of WRN for DNA is not responsible for the lack of exonuclease activity. WRN exonuclease is distinguished, therefore, from the large majority of the known nucleases by its preference for recessed DNA in a partial duplex (see "Discussion").

WRN Exonuclease Is Stimulated by Nucleoside Triphosphates—Since the helicase activity of WRN is completely dependent on the hydrolysis of NTPs, we tested whether the activity of WRN exonuclease is similarly affected by nucleoside triphosphates. A partial duplex of $^{32}$P-labeled 5'-20-mer/unlabeled 46-mer was incubated with 15 or 30 fmol of WRN protein in the absence or presence of 1 mM of each of the indicated nucleoside triphosphates. Following a 10-min incubation at 37 °C, the nucleolytically fragmented $^{32}$P-labeled 5'-20-mer was resolved from undigested DNA by electrophoresis through a denaturing polyacrylamide gel as shown in Fig. 2. Relative amounts of hydrolyzed DNA were quantitated by PhosphorImager analysis.

<table>
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<tr>
<th>Nucleoside phosphate</th>
<th>$X$-fold stimulation of DNA hydrolysis</th>
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<tr>
<td></td>
<td>15 fmol WRN</td>
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<tr>
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<tr>
<td>ATP</td>
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* The fraction of $^{32}$P-labeled 5'-20-mer that was degraded by 15 and 30 fmol of WRN nuclease in the absence of nucleoside phosphate was 1.1 and 6.1%, respectively.

Table II

Effect of nucleoside phosphates on DNA hydrolysis by WRN exonuclease

WRN protein (15 or 30 fmol) was incubated with the $^{32}$P-labeled 5'-20-mer/unlabeled 46-mer partial DNA duplex in the absence or presence of 1 mM of each of the indicated nucleoside phosphates at 37 °C for 10 min. Products of nucleolytic digestion were resolved by electrophoresis through a 14% polyacrylamide-urea gel as shown in Fig. 2. Relative amounts of hydrolyzed DNA were quantitated by PhosphorImager analysis.
WRN Protein Digests DNA Exonucleolytically with a 3' → 5' Directionality—To determine the polarity of DNA digestion by the WRN nuclease, increasing amounts of the protein were incubated with a partial duplex of unlabeled 46-mer DNA annealed to the 3'-end (Fig. 3, upper left and right panels, respectively). By contrast, no such intermediate degradation products were detected when the 3'-32P-labeled DNA was incubated with WRN; the only product that accumulated proportionally to the amount of enzyme or time of incubation was a single 32P-labeled dCMP residue removed from the 3'-terminus (Fig. 3, lower left and right panels, respectively). The progressive accumulation of 5'-labeled but not 3'-labeled DNA fragments indicates that WRN does not digest DNA endonucleolytically and that it acts exclusively as a 3' → 5' exonuclease under our experimental conditions.

**WRN Exonuclease Preferentially Digests Recessed DNA with a Single 3'-Mismatched Nucleotide**—The sequence of the WRN exonuclease is homologous to the 3'-5'-proofreading domain of E. coli DNA polymerase I (22, 23). We therefore examined whether the 3' → 5' WRN exonuclease is capable of removing non-complementary nucleotides from the 3'-end of DNA. WRN nuclease was incubated with DNA substrates that were comprised of the unlabeled 46-mer annealed to either a 5'-32P-labeled fully complementary 23-mer, a 24-mer with a single 3'-terminal mismatch, or a 25-mer with two mismatches at the 3'-terminus (see Table I). Under our experimental conditions, removal of the single 3'-terminal mismatched nucleotide was more efficient than that of a complementary terminal base.

**WRN Exonuclease Digests DNA to Generate 5'-dNMP Residues**—To determine whether WRN nuclease cleaves DNA to generate 3'- or 5'-dNMP residues, a 21-mer DNA substrate 32P-labeled at its 3'-terminal cytosine residue was hydrolyzed by increasing amounts of WRN or with a constant amount of the enzyme for different periods of time. Aliquots of the reaction mixtures were co-chromatographed through a polyethyleneimine-cellulose thin layer with 3'- and 5'-dCMP markers (see “Experimental Procedures”). As seen in Fig. 4, the amount of [32P]dCMP accumulated was proportional to the amount of added WRN and to the duration of DNA hydrolysis. The absence of products other than free dCMP is in accord with the 3' → 5' directionality of DNA digestion (Fig. 3, lower left and right panels) and with the inability of WRN to cleave DNA exonucleolytically. Further, the 32P-labeled dCMP product of WRN nuclease co-migrated with the 5'-dCMP marker but not with the 3'-dCMP marker (Fig. 4) indicating that WRN cleaves phosphodiester bonds to generate 5'-dNMP residues.

**WRN Exonuclease Digests DNA Molecules with Either a 3'-OH or a 3'-PO4 Terminus**—To examine the relative capacity of WRN nuclease to degrade DNA molecules that terminate with either a 3'-OH or a 3'-PO4 group, the enzyme was incubated with increasing concentrations of a hybrid of the 46-mer DNA and a 32P-labeled 5'-20-mer that ends with either a 3'-OH or 3'-PO4 terminus (Table I). Both DNA substrates were displaced equivalently by WRN helicase (results not shown). Likewise, the 3'-OH and 3'-PO4 oligomers were degraded to similar extents by WRN exonuclease. PhosphorImager analysis and Lineweaver-Burk plots of the amount of DNA degraded as a function of substrate concentration yielded similar kinetic constants for both substrates. The Vmax values were 0.02 and 0.3 pmol of DNA digested/10 min, and the K values were 13 × 10⁻⁵ and 6 × 10⁻⁹ s for the 3'-OH 20-mer and 3'-PO4 20-mer, respectively (Fig. 5). The WRN 3' → 5' exonuclease is further distinguished by its ability to digest DNA that has a 3'-PO4 terminus equally or in a slight preference over DNA that carries a 3'-OH end.

**WRN Exonuclease Preferentially Digests Recapped DNA with a Single 3'-Mismatched Nucleotide**—The sequence of the WRN exonuclease is homologous to the 3' → 5' proofreading domain of E. coli DNA polymerase I (22, 23). We therefore examined whether the 3' → 5' WRN exonuclease is capable of removing non-complementary nucleotides from the 3'-end of DNA. WRN nuclease was incubated with DNA substrates that were comprised of the unlabeled 46-mer annealed to either a 5'-32P-labeled fully complementary 23-mer, a 24-mer with a single 3'-terminal mismatch, or a 25-mer with two mismatches at the 3'-terminus (Table I). Under our experimental conditions, removal of the single 3'-terminal mismatched nucleotide was more efficient than that of a complementary terminal base.
pair. However, 3’-terminal double mismatches were relatively resistant to digestion by WRN (Fig. 6). A similar preference for DNA containing a single mismatch was observed (data not presented) when the reaction mixtures were incubated with WRN-DH, a mutant enzyme that expresses only the N-terminal exonuclease domain (16). These results suggest that, at least within the sequence and structure contexts of the DNA substrates examined, WRN exonuclease preferentially hydrolyzes DNA with a single 3’-terminal mismatched nucleotide.

DISCUSSION

In the preceding paper (16), we demonstrated that WRN possesses an integral exonuclease activity. The present study characterizes this activity. Using a >90% homogeneous preparation of recombinant wild-type WRN, we have identified several novel properties of the exonuclease that distinguish it from most known eukaryotic nucleases.

3’-5’ exonuclease activities are, in general, small molecular weight proteins that hydrolyze DNA in an ATP-independent manner (24). The majority of these enzymes exclusively digest single-stranded, rather than double-stranded, DNA. The catalytic properties of the 3’→5’ WRN exonuclease are markedly different. First, the WRN exonuclease is a large molecular weight entity (16). Apart from the 3’→5’ proofreading exo- nuclease that is intrinsic to eukaryotic DNA polymerases δ and ε (24), no other proteins of ~160 kDa have thus far been reported to possess nuclease activity. Second, the WRN exonuclease preferentially degrades a recessed strand in a partial DNA duplex to generate 5’-deoxynucleoside monophosphates (Figs. 1 and 4). Unlike most other 3’→5’ exonucleases, it fails to measurably hydrolyze single-stranded DNA, a blunt-ended DNA duplex or a 3’-protruding DNA strand of a partial duplex when used over a 100-fold range of DNA concentrations (Fig. 1). The fact that the helicase activity of WRN unwinds the duplex with the protruding DNA strand argues that the lack of hydrolysis is not simply due to a reduced binding affinity of WRN for these DNA molecules. Also, the absence of nucleolytic degradation of single-stranded DNA (Fig. 1) negates the presence of a nonspecific DNase in the preparation. Recessed DNA chains terminating with either a 3’-OH or a 3’-PO₄ group are equally degraded by the enzyme, as reflected by the similar values of Vₘₐₓ and Kₘₐₜ for both DNA substrates (Fig. 5). Furthermore, 3’ recessed termini with either a matched sequence or with a single mismatch are preferably degraded over those with two mismatches (Fig. 6). Finally, the exonuclease activity of WRN is markedly stimulated by ATP or dATP at low enzyme concentrations; CTP, dCTP, UTP, and dTTP also increase the activity (Fig. 2 and Table II). The most likely explanation for the stimulatory effect is that binding and hydrolysis of NTPs in the helicase domain induces a conformational change of the nuclease domain that, in turn, increases its exonuclease activity.

Another unique characteristic of the WRN exonuclease is its...
low processivity, as reflected by the appearance of intermediate sized degradation products (Figs. 1, 2, 3, and 6). That this is not a result of pause sites in the sequence of the oligonucleotide was shown using a 20-mer with a different sequence context; a similar pattern of major degradation products, ranging from 14–19 nucleotides in length, is observed (data not presented). The low processivity is also indicated by the high enzyme concentrations and the extended incubation times that are required to see the appearance of the single 5′-terminal nucleoside monophosphate (Figs. 2 and 3), the completely digested product of the WRN nuclease. However, the processivity could be increased in vivo by the interaction of WRN with accessory proteins. It is possible that the primary function of the WRN exonuclease is the hydrolysis of short stretches of DNA. Alternatively, competing helicase and exonuclease activities may limit the processivity of the WRN exonuclease.

Several of the properties of the WRN exonuclease are similar to those of E. coli exonuclease III (exo III). Both nuclease preferentially hydrolyze recessed DNA with a 3′ → 5′ polarity (Fig. 3 and Ref. 25). Additionally, both proteins are 3′-phosphomonoesterases, capable of hydrolyzing a DNA chain terminating with a 3′-PO₄ group (Fig. 5 and Ref. 26). However, while E. coli exo III is an endonuclease responsible for the repair of apurinic-apyrimidinic sites in DNA (27), the WRN nuclease does not exhibit endonucleolytic activity with any of the substrates used in this study (Fig. 1).

The exonuclease activity of E. coli exo III has been postulated to play a role in the repair of DNA damaged by reactive oxygen species. This proposal is based on the observation that bacteria lacking functional exo III show an increased sensitivity to H₂O₂ (28). The hypersensitivity of WS cells to 4NQO (29), an agent also known to generate O₂ free radicals, may imply that the exonuclease activity of WRN is required for repair of DNA damaged by 4NQO. 4NQO forms direct adducts with DNA and is exo III hypersensitive for repair of DNA damaged by 4NQO forms direct adducts with DNA and exonuclease is the hydrolysis of short stretches of DNA. Alternatively, competing helicase and exonuclease activities may limit the processivity of the WRN exonuclease.

It is more conceivable, however, that the two activities of WRN are manifested at different steps of the same pathway. In our accompanying paper (16) we showed that DNA unwinding does not require the exonuclease function and vice versa. In this and other studies (21), we have demonstrated that whereas DNA unwinding is completely dependent on the hydrolysis of NTPs, exonuclease digestion is not. These observations suggest that the two enzymatic activities of WRN need not function concertedly.

Based on the known DNA replication phenotypes of WS cells (14, 15), WRN may participate in DNA synthesis. In this process, the WRN exonuclease may remove a 3′-terminal nucleoside that is misincorporated by a DNA polymerase lacking an associated 3′ → 5′ proofreading activity. The WRN helicase on the other hand could function in lagging strand DNA synthesis by displacing upstream Okazaki fragments and enabling FEN1/RTH1 endonuclease to digest the displaced strand and DNA ligase to join the DNA. Alternatively, the restricted sensitivity of WS cells to 4NQO (29) suggests that WRN may be required to repair DNA modified by 4NQO. Among other alterations, 4NQO generates chromosomal breaks that, if repaired incorrectly, can lead to chromosome translocations and other types of genetic instability. Since WS cells are characterized by this form of genomic instability, it is tempting to speculate that one function of WRN may be the repair of DNA double strand breaks. This pathway could require the participation of a 3′ → 5′ exonuclease to remove PO₄ groups from the 3′-terminus and/or create a gap, and a helicase to unwind DNA during repair synthesis (30). Model in vitro systems that mimic partial steps of DNA replication, repair, and recombination (31–33) should allow one to assess the ability of WRN to function as a helicase and an exonuclease in these processes.

REFERENCES

Werner Syndrome Protein: II. CHARACTERIZATION OF THE INTEGRAL 3′ → 5′ DNA EXONUCLEASE
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