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Werner Syndrome Protein

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 stimulated by ATP, dATP, or CTP. WRN exonuclease digests DNA with a 3′ → 5′ directionality to generate 5′-dNMP products, and DNA strands terminating with either a 3′-OH or 3′-PO_4 group are hydrolyzed to similar extents. A recessed DNA strand with a single 3′-terminal mismatch is hydrolyzed more efficiently by WRN than one with a complementary nucleotide, but the enzyme fails to hydrolyze 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′-PO_4 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, 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 nuclear 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′-deoxynucleoside monophosphate products, 2) its preferred substrate is a recessed strand of a partial DNA duplex that terminates with either a 3′-OH or 3′-PO_4 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-^{32}P]ATP and [α-^{32}P]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 oligodeoxynucleotides 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 ^{32}P 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 [^{32}P]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 $MgCl_2$, 40 mM KCl, 5 μM dCTP, 5 μCi of [α-^{32}P]dCTP, 10 μg of bovine serum albumin, 10 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 [^{32}P]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 ^{32}P-labeled 3′-21-mer

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[1] The abbreviations used are: WS, Werner syndrome; DTT, dithiothreitol; ATPγS, adenosine 5′-*O*-(thiotriphosphate); 4NQO, 4-nitroquinoline 1-oxide.

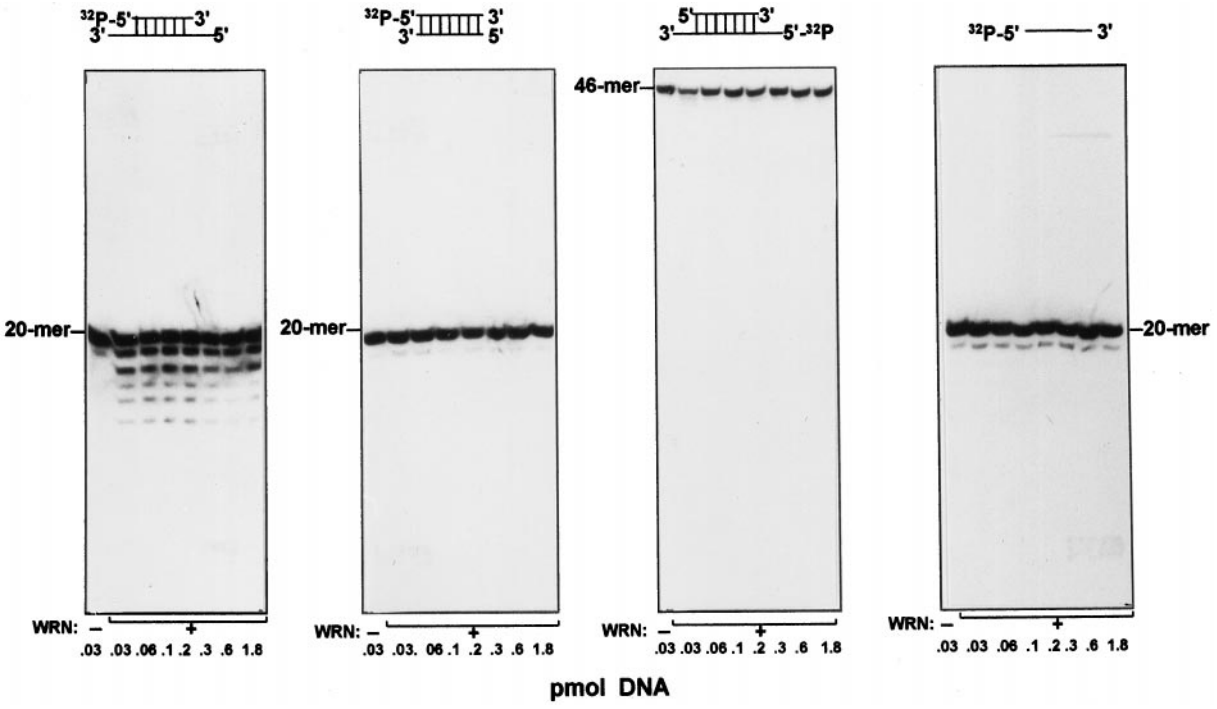

FIG. 1. **WRN exonuclease selectively hydrolyzes the recessed strand in a partial DNA duplex.** WRN protein (15 fmol) was incubated with a mixture of 0.03 pmol of each 5′-^{32}P-labeled DNA substrate and increasing amounts of the same unlabeled DNA molecule at 37 °C for 10 min. Control reactions containing each of the ^{32}P-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 ^{32}P-labeled 5′-20 mer and unlabeled 46-mer; blunt-ended duplex of ^{32}P-labeled 5′-20 mer and unlabeled anti-20-mer; partial duplex of ^{32}P-labeled 5′-46-mer and unlabeled 20 mer; single-stranded ^{32}P-labeled 5′-20 mer.

TABLE I
DNA oligomers used in this study

Oligomer	Nucleotide sequence
46-mer	5′-d(GCGCGGAAGCTTGGCTGCAGAATATTGCTAGCGGGAAATCGGCGCG)-3′
20-mer	5′-d(CGCTAGCAATATTCTGCAGC)-3′
20-mer 3′-P	5′-d(CGCTAGCAATATTCTGCAGC)-3′-PO_4
Anti-20-mer	5′-d(GCTGCAGAATATTGCTAGCG)-3′
23-mer	5′-d(CGCGCCGAATTCCCGCTAGCAAT)-3′
24-mer	5′-d(CGCGCCGAATTCCCGCTAGCAATG)-3′
25-mer	5′-d(CGCGCCGAATTCCCGCTAGCAATGC)-3′

DNA band was excised, extracted from the gel, and precipitated by ethanol (20); the isolated DNA was reannealed to the unlabeled complementary 46-mer oligonucleotide as described above.

Assays for WRN Exonuclease Activity—WRN protein purified to near homogeneity by successive steps of ion exchange and affinity chromatography steps (16) was used throughout this study. In a standard assay for exonuclease activity, WRN was incubated with a ^{32}P-labeled 5′-20-mer/46-mer partial duplex substrate (Table I) under the previously described DNA helicase/exonuclease reaction conditions (16). Substrate specificity of the exonuclease was measured using the standard DNA substrate as well as the following oligomers: single-stranded ^{32}P-labeled 5′-20-mer; blunt-ended ^{32}P-labeled 5′-20-mer/unlabeled anti 20-mer duplex, and a 20-mer/^{32}P-labeled 5′-46-mer partial duplex. To measure the relative capacity of WRN exonuclease to digest matched and terminally mismatched DNA substrates, unlabeled 46-mer DNA was annealed to either a fully matched ^{32}P-labeled 5′-23-mer, a 3′-singly mismatched 24-mer, or a 3′-doubly mismatched 25-mer (Table I). Products of exonucleolytic digestion of DNA were resolved by electrophoresis through a 14% polyacrylamide-urea gel and were visualized by autoradiography or quantitated by PhosphorImager analysis.

Analysis of the Products of DNA Hydrolysis by WRN—To determine whether hydrolysis of DNA by WRN nuclease generates 3′- or 5′-dNMP residues, ^{32}P-labeled 3′-21-mer in a partial duplex with unlabeled 46-mer was digested by WRN, and the labeled nucleoside monophosphate released was resolved and identified by thin layer chromatography. The DNA substrate was incubated with either an invariable amount of WRN protein (0.25 pmol) for different periods of time or with increasing amounts of WRN (0.125–1.5 pmol) for 10 min at 37 °C. DNA hydrolysis was terminated by setting the reaction mixtures on ice, and 50 nmol each of 3′-dCMP and 5′-dCMP marker nucleoside monophosphates were added. Aliquots were spotted on a polyethyleneimine-Cellulose F thin layer plate, and 3′- and 5′-nucleoside monophosphates were resolved by chromatography using a mixture of isobutyric acid:H_2O:NH_4OH (66:20:3). A wick of Whatman No. 3MM filter paper was attached to the top of the plate to increase the distance of migration and the resolution between 3′- and 5′-dCMP. Spots of migrated 3′- and 5′-dCMP standards were visualized by UV light, and ^{32}P-labeled dCMP was located by autoradiography.

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 ^{32}P-labeled DNA substrates was mixed with increasing amounts of the same unlabeled DNA and incubated with WRN as described. Measurements of the exonucleolytic 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

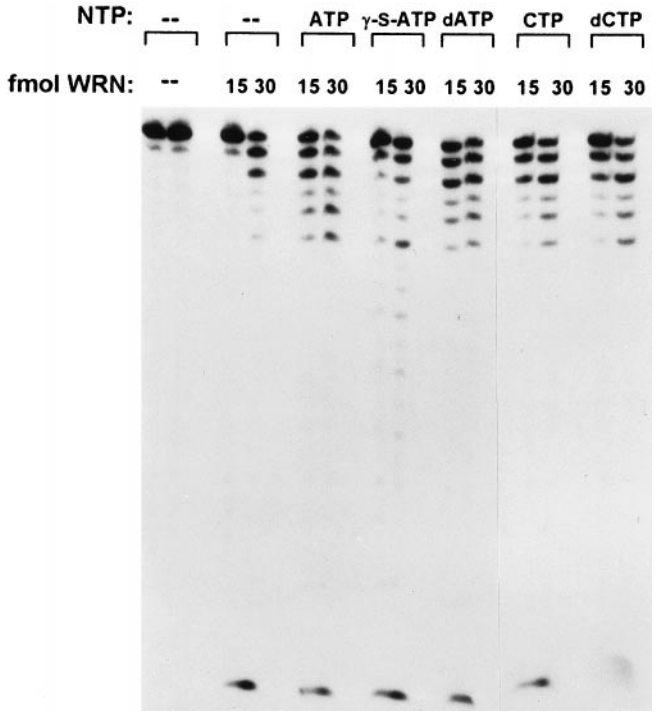

FIG. 2. **WRN exonuclease is stimulated by nucleoside triphosphates.** A partial duplex of ^{32}P-labeled 5′-20-mer annealed to unlabeled 46-mer DNA was mixed with 15 or 30 fmol of WRN protein in the absence or presence of 1 mM each of the indicated nucleoside phosphates. Following a 10-min incubation 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 described.

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.

Nucleoside phosphate	*X*-fold stimulation of DNA hydrolysis	
	15 fmol WRN	30 fmol WRN
None[a]	1.0	1.0
ATP	4.55	1.9
ATPγS	1.35	0.85
dATP	4.65	1.5
AMP	1.5	0.6
cAMP	1.0	0.8
GTP	1.35	1.05
dGTP	1.8	1.2
CTP	3.1	1.3
dCTP	1.6	1.4
UTP	2.7	1.25
dTTP	2.7	0.95

[a] 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.

value of 22×10^{-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,

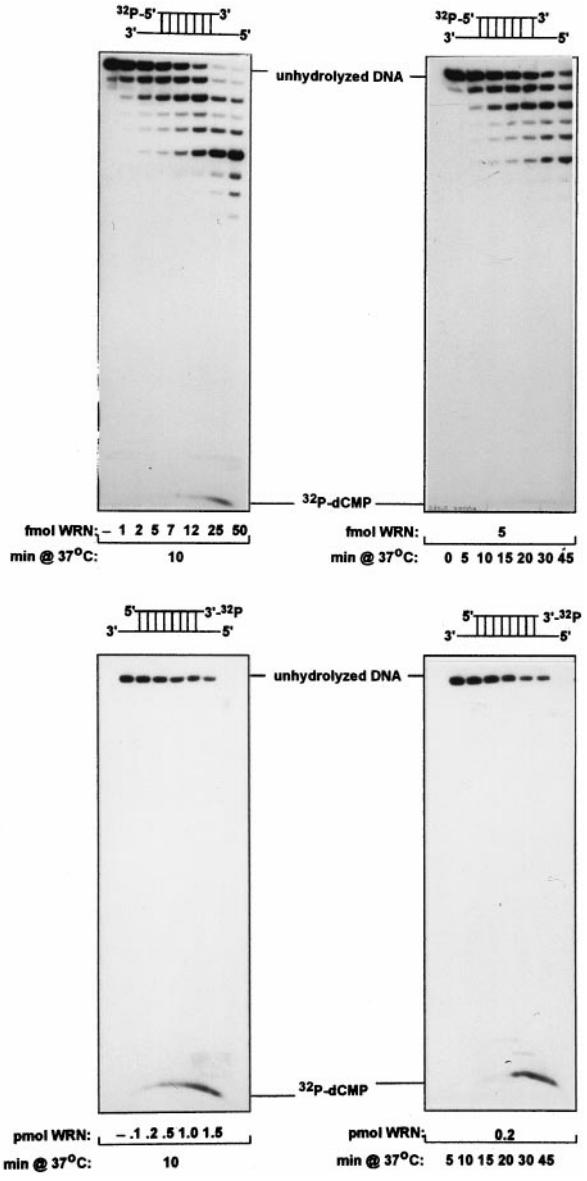

FIG. 3. **WRN nuclease hydrolyzes DNA with a 3′ → 5′ polarity.** Partial DNA duplexes of unlabeled 46-mer and either ^{32}P-labeled 5′-20-mer (*upper left* and *right panels,* respectively) or ^{32}P-labeled 3′-21-mer (*lower left* and *right panels,* respectively) were incubated with the indicated amounts of WRN for 10 min or with 5 fmol of WRN for the indicated periods of time at 37 °C. Products of DNA hydrolysis were resolved by electrophoresis through a 14% polyacrylamide-urea gel.

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 different nucleoside tri- or monophosphates as indicated. In accord with recently reported results (21), we found that the activity of WRN helicase was entirely dependent on ATP hydrolysis and that dATP, CTP, or dCTP could substitute for ATP to a significant extent (data not shown). On the other hand, WRN DNA helicase activity was

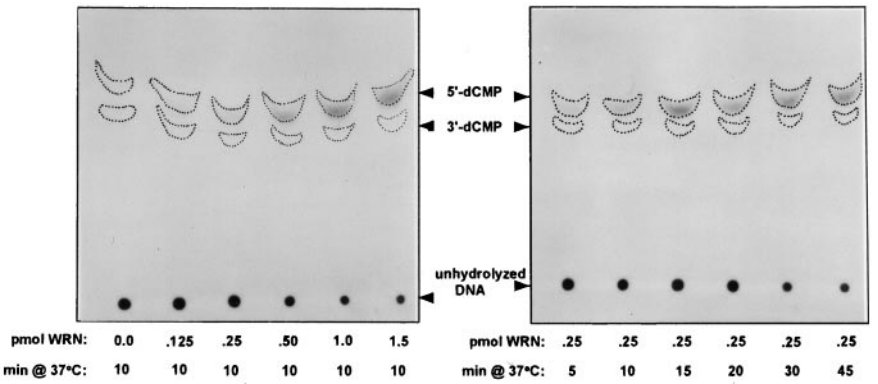

FIG. 4. **WRN protein cleaves DNA to generate 5′-dNMP residues.** A partial DNA duplex of ^{32}P-labeled 3′-21-mer annealed to unlabeled 46-mer was incubated with the indicated amounts of WRN protein and for the denoted periods of time at 37 °C. Following termination of DNA hydrolysis, 3′- and 5′-dCMP markers were added to the reaction mixtures, and aliquots were chromatographed through a polyethyleneimine-cellulose thin layer. Positions of the 3′ and 5′ markers, as visualized by UV light, are marked by *dotted lines* and indicated by *arrows*, respectively.

undetectable in the presence of nonhydrolyzable ATPγS, AMP, cAMP, GTP, dGTP, cGMP, UTP, or dTTP (results not shown). By contrast, hydrolysis of DNA by WRN exonuclease was observed in the absence of NTPs; this activity could be further increased by hydrolyzable NTPs. Fig. 2 shows a representative electrophoretogram of the DNA digestion products generated by WRN nuclease in the presence of different nucleoside phosphates, and Table II summarizes the quantitative results of the extents of DNA degradation. Under our experimental conditions, 15 and 30 fmol of WRN degraded 1.1% and 6.1% of the DNA substrate (100 fmol), respectively, in the absence of NTPs. Selected nucleoside triphosphates were especially effective in stimulating DNA hydrolysis by the lower amount of WRN nuclease. For example, ATP and dATP stimulated hydrolysis of the 20-mer DNA substrate by 4- to 5-fold. By contrast, although non-hydrolyzable ATPγS appeared to increase the processivity of the WRN exonuclease, it did not stimulate the overall extent of digestion (Table II). Like the WRN DNA helicase (21), WRN exonuclease was also stimulated by CTP and dCTP. Interestingly, whereas UTP or dTTP did not increase the activity of WRN helicase (results not shown), they did enhance the nuclease activity ~3-fold using 15 fmol of WRN protein (Table II). Thus, unlike WRN DNA helicase that completely depends on ATP hydrolysis, its nuclease activity was measurable in the absence of hydrolyzable nucleoside triphosphates. However, at protein to DNA molar ratios of <0.3, WRN-catalyzed DNA hydrolysis was significantly stimulated by selected nucleoside triphosphates in the order ATP = dATP > CTP > UTP = dTTP > dGTP = dCTP.

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 either a ^{32}P-labeled 5′-20-mer or a ^{32}P-labeled 3′-21-mer for 10 min at 37 °C. In a parallel experiment, a constant amount of WRN protein (5 fmol) was incubated with each DNA substrate for different periods of time. Whereas both substrates were equally unwound by WRN helicase (data not shown), there was a difference in the profiles of degradation products that accumulated with either 5′- or 3′-labeled DNA. Incubation of WRN with the 5′-^{32}P-labeled DNA substrate resulted in the removal of single nucleotide residues to generate a ladder of progressively shortened DNA fragments, all labeled at the 5′-end (Fig. 3, *upper left* and *right panels,* respectively). By contrast, no such intermediate degradation products were detected when the 3′-^{32}P-labeled DNA was incubated with WRN; the only product that accumulated proportionally to the amount of enzyme or time of incubation was a single ^{32}P-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 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 ^{32}P-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 [^{32}P]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 endonucleolytically. Further, the ^{32}P-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 Protein Degrades DNA Molecules with Either a 3′-OH or a 3′-PO_4 Terminus—To examine the relative capacity of WRN nuclease to degrade DNA molecules that terminate with either a 3′-OH or a 3′-PO_4 group, the enzyme was incubated with increasing concentrations of a hybrid of the 46-mer DNA and a ^{32}P-labeled 5′-20-mer that ends with either a 3′-OH or 3′-PO_4 terminus (Table I). Both DNA substrates were displaced equivalently by WRN helicase (results not shown). Likewise, the 3′-OH and 3′-PO_4 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 V_{max} values were 0.03 and 0.02 pmol of DNA digested/10 min, and the K_m values were 13×10^{-9} and 6×10^{-9} M for the 3′-OH 20-mer and 3′-PO_4 20-mer, respectively (Fig. 5). The WRN 3′ → 5′ exonuclease is further distinguished by its ability to digest DNA that has a 3′-PO_4 terminus equally or in a slight preference over DNA that carries a 3′-OH end.

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′-^{32}P-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

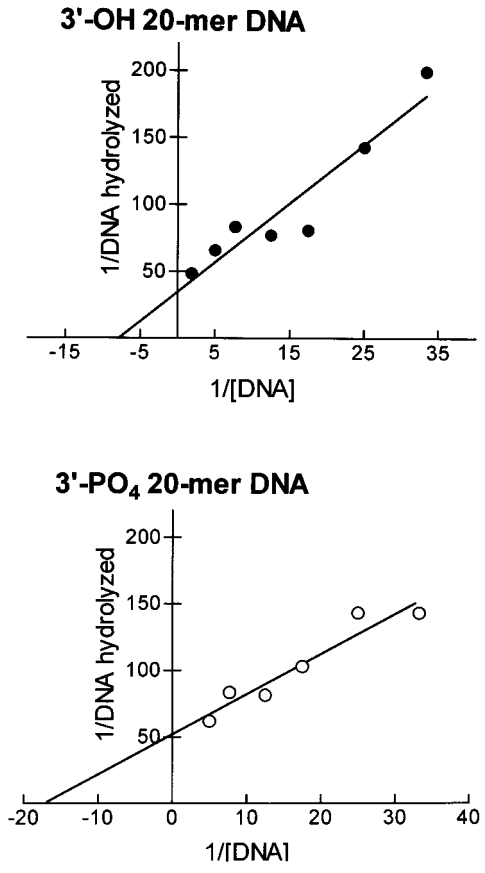

FIG. 5. **WRN exonuclease digests recessed 20-mer DNA with either a 3′-OH or a 3′-PO_4 terminus to a similar extent.** WRN nuclease (15 fmol) was incubated with increasing concentrations (3–53 nmol) of either ^{32}P-labeled 5′-20-mer DNA or ^{32}P-labeled 5′-20-mer 3′-P DNA (Table I) for 10 min at 37 °C. Products of DNA digestion were resolved by electrophoresis, and extents of DNA hydrolysis were quantitated by PhosphorImager analysis. The kinetic constants were derived from Lineweaver-Burk plots.

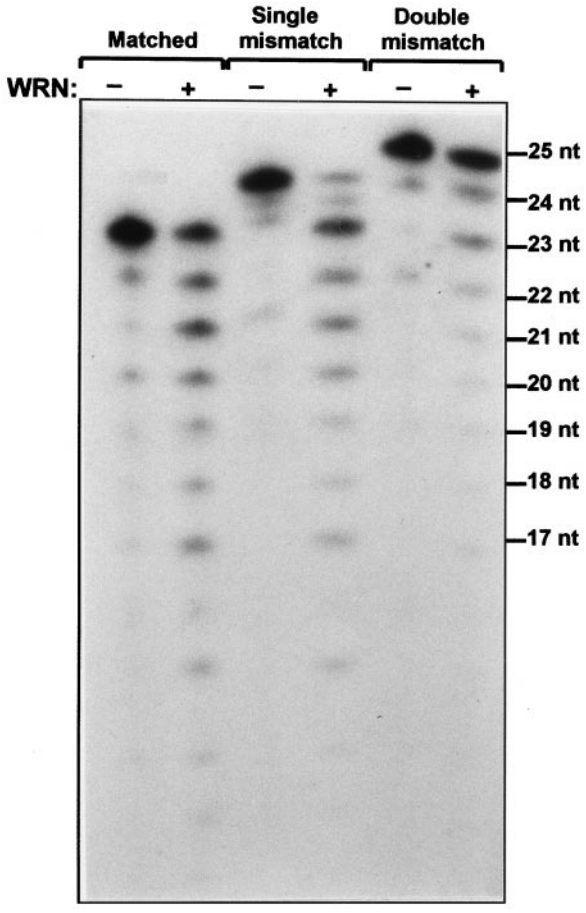

FIG. 6. **Hydrolysis of a recessed DNA strand with matched and singly or doubly mismatched 3′-terminal nucleotides by WRN exonuclease.** Partial duplexes (0.1 pmol) of unlabeled 46-mer and ^{32}P-labeled 5′-complementary 23-mer or a 24-mer with a single 3′-terminal mismatch, or a 25-mer with two 3′-terminal mismatches, were incubated in the absence or presence of 30 fmol of WRN protein. Products of DNA degradation were resolved by electrophoresis. Nucleotide sequences of the respective oligomers are listed in Table I. The sizes of the fragmented DNA products were determined using labeled DNA oligonucleotides of known lengths.

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-ΔH, 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′ exodeoxyribonucleases 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 exonucleases that are 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 3′-PO_4 group are equally degraded by the enzyme, as reflected by the similar values of V_{max} and K_m 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 exonucleolytic 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 nucleases 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′-PO_4 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 H_2O_2 (28). The hypersensitivity of WS cells to 4NQO (29), an agent also known to generate O_2 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 also induces the formation of reactive oxygen species that modify DNA and generate double strand breaks that could result in recessed DNA strands with either a 3′-OH or 3′-PO_4 terminus. Thus, like exo III, WRN exonuclease may be involved in the repair of oxygen-damaged DNA.

The identification and characterization of the novel WRN exonuclease raises an interesting question. Do the helicase and exonuclease activities function together? If they do, the opposite polarities of DNA unwinding and hydrolysis make the process highly unusual. At least two models can be invoked to explain these inconsistencies. In the first, WRN binds the 3′ tail of a DNA chain and initiates unwinding of the complementary annealed strand. When it approaches the last few bases of annealed DNA, it is unable to proceed further. At this point, WRN undergoes a conformational change that orients the nuclease domain in proximity to the 3′ end of the strand that is being displaced, promoting the exonucleolytic degradation rather than unwinding of the terminally base-paired sequences. The second model proposes that the DNA, rather than WRN, assumes an altered conformation. For example, if the DNA strand that is bound by WRN were to bend, WRN could simultaneously unwind DNA from one end and hydrolyze it from the other.

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, exonucleolytic 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 nucleotide 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 PO_4 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.

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