DNase I Footprinting and Enhanced Exonuclease Function of the Bipartite Werner Syndrome Protein (WRN) Bound to Partially Melted Duplex DNA*

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Werner syndrome is a premature aging and cancer-prone hereditary disorder caused by deficiency of the WRN protein that harbors 3'→5' exonuclease and RecQ-type 3'→5' helicase activities. To assess the possibility that WRN acts on partially melted DNA intermediates, we constructed a substrate containing a 21-nucleotide noncomplementary region asymmetrically positioned within a duplex DNA fragment. Purified WRN shows an extremely efficient exonuclease activity directed at both blunt ends of this substrate, whereas no activity is observed on a fully duplex substrate. High affinity binding of full-length WRN protects an area surrounding the melted region of the substrate from DNase I digestion. ATP binding stimulates but is not required for WRN binding to this region. Thus, binding of WRN to the melted region underlies the efficient exonuclease activity directed at the nearby ends. In contrast, a WRN deletion mutant containing only the functional exonuclease domain does not detectably bind or degrade this substrate. These experiments indicate a bipartite structure and function for WRN, and we propose a model by which its DNA binding, helicase, and exonuclease activities function coordinately in DNA metabolism. These studies also suggest that partially unwound or noncomplementary regions of DNA could be physiological targets for WRN.

The RecQ helicases are a family of proteins that catalyze the ATP-dependent unwinding of double-stranded nucleic acids (reviewed in Refs. 1 and 2). There are genes coding for at least five RecQ family members (RECQL, BLM, WRN, RECQ4/RTS, and RECQ5) in the human genome that are highly homologous to one another only within seven distinct sequence motifs that comprise a conserved helicase domain. The hereditary diseases known as Bloom, Werner, and Rothmund-Thomson syndromes are the result of the loss of function of BLM, WRN, or RTS, respectively (3–5). Although genomic instability is common to each of these diseases, the overall phenotype of each disease is different, indicating that the functions of BLM, WRN, and RTS are distinct or at least partially nonoverlapping. Interestingly, WRN is the only human RecQ protein to have an exonuclease domain in addition to the helicase domain (6). The Werner syndrome (WS) phenotype is characterized by early onset of a number of aging characteristics, including graying and loss of hair, increased wrinkling and ulceration of the skin, osteoporosis, atherosclerosis, and an increased frequency of age-related maladies such as cancer, diabetes, and cataracts. WRN-deficient cells from WS patients have elevated levels of genomic instability typified by increased deletions, insertions, and translocations (7, 8), as well as an accelerated rate of telomere loss (9, 10). Both the WS cellular phenotype and the WRN primary amino acid sequence point to a role in DNA metabolism that, when absent, results in large scale genetic change.

A number of laboratories (including ours) have successfully overexpressed and purified a recombinant WRN protein, and its basic catalytic activities have been characterized. Consistent with the presence of ATPase/helicase amino acid sequence motifs in the central region of the protein, WRN is a DNA-dependent ATPase (11). In conjunction with ATP hydrolysis, WRN unwinds DNA-DNA and DNA-RNA duplexes that contain a single-stranded region 3' to the duplex to be unwound, i.e. 3'→5' directionality (11–14). More recently, WRN has also been shown to disrupt DNA triplexes (15) and certain G-quadruplex structures (16). WRN helicase can apparently carry out branch migration of Holliday structures as well (17). In general, studies on WRN, BLM, and Sgs1 (the lone RecQ homolog in Saccharomyces cerevisiae) have shown remarkable similarities in helicase function and DNA substrate specificity (16, 18–24).

WRN has also been shown to be an exonuclease (25–27), consistent with the presence of RNase D-type nuclease domains in the N-terminal region of the protein (6). This activity is directed to the 3' end of a duplex substrate (3'→5' directionality) preferably containing a 5' overhang (recessed 3' end) and has not been observed on single-stranded DNA or double-stranded DNA with blunt ends or 3' overhangs (25, 28). On DNA substrates tested thus far, WRN exonuclease activity is dramatically stimulated by ATP hydrolysis (28), suggesting some cooperativity between the ATPase and exonuclease functions of WRN. However, exonuclease activity can be observed in the absence of ATP (28), and mutant WRN proteins lacking either ATPase/helicase activity or the entire helicase domain still retain exonuclease activity (27), suggesting that the exonuclease and helicase domains are functionally independent and, in all likelihood, physically separate. Importantly, the absence of exonuclease activity in the other human RecQ homologs suggests that the unique WS phenotype may be in part the result of loss of the specific exonuclease function of WRN.

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The abbreviations used are: WS, Werner syndrome; ATP, adenine 5'-O-(3-thiotriphosphate); BSA, bovine serum albumin; DNase I, bovine pancreatic deoxyribonuclease I; EMSA, electrophoretic mobility shift assay; nt, nucleotide(s).
In this study, a defined DNA substrate containing an internal region of noncomplementarity was designed to closely approximate the localized melting of DNA duplexes that occurs during many metabolic processes. The DNA binding affinity and catalytic activities of recombinant wild type and mutant WRN proteins were examined on this substrate and compared with other model DNA substrates. We have demonstrated that, when compared with completely complementary substrates, a DNA substrate containing 21 unpaired nucleotides (nt) in both strands has extremely high affinity for the WRN protein. We have further characterized this binding and the effect of nucleotide cofactors by deoxyribonuclease I (DNase I) footprinting. We show that ATP hydrolysis is not required for high affinity binding of WRN to the melted region, and that the exonuclease domain does not bind stably to this substrate. Nevertheless, the WRN exonuclease function is highly active on this bubble substrate, and this activity is both independent of ATP binding or hydrolysis and further unwinding by WRN helicase function.

Our results indicate coordination between the DNA binding and exonuclease domains of WRN and suggest that a non-complementary (heteroduplex) or melted region of DNA could be the physiological substrate of WRN. Such structures may be indicative of DNA replication, recombination, or repair intermediates formed in vivo that require processing by WRN helicase and/or exonuclease activity.

**EXPERIMENTAL PROCEDURES**

**WRN Purification**—Recombinant wild type and mutant WRN proteins were overexpressed and purified essentially as described previously (29). The WRN-K577M mutant contains a lysine to methionine point mutation at amino acid residue 577 in motif I of the conserved helicase domain; this protein lacks both ATPase and helicase activities (11, 12). The WRN-E84A mutant contains a glutamate to alanine point mutation at amino acid residue 577 in motif I of the conserved helicase domain that abolishes exonuclease activity (29). The WRN-K577M–1432 protein contains only the N-terminal 368 amino acids of the full-length protein; however, this truncated protein possesses the entire conserved exonuclease domains and retains exonuclease activity (27). The activities of wild type and mutant WRN proteins used in this study are summarized in Table I. All constructs contained an N-terminal hexahistidine tag to facilitate purification by Ni2+–NTA chromatography affinity chromatography. The WRN plasmid DNA was isolated from insect cells (Sf9 strain) infected with baculovirus containing WRN cDNA sequences at an multiplicity of infection of 10 and harvested 72 h at 4°C. The wild type, WRN-K577M, and WRN-E84A proteins were purified by sequential liquid chromatographic steps using DEAE-Sepharose, Q-Sepharose, and nickel-nitrioltriacetic acid-garose resins (29). The purification of WRN-K577M–1432 was modified slightly. After cell lysis, WRN-K577M–1432 protein was bound to DEAE-Sepharose in 150 m M Tris-HCl, pH 8.0, and quantitation were accomplished using a Storm 860 phosphorimaging system and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The band intensities (minus background) for the double-stranded and single-stranded DNA species were determined for WRN-treated and untreated reactions. Percentage of unwinding is calculated as the ratio (100) of single-stranded species to the total amount of substrate in WRN-treated reactions, including subtraction of the low percentage of single-stranded species in untreated controls.

**Exonuclease Assay**—In WRN reaction buffer either without ATP or including ATP or ATP-S (1 mM) where indicated, 32P-labeled DNA substrates (~0.5 femol each) were incubated for 1 h at 37°C with wild type or mutant WRN protein (2–600 fmol). The reactions were stopped by addition of an equal volume of formamide loading buffer (95% formamide, 20 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol), and the DNA products were denatured at 90°C for 5 min and separated by electrophoresis on a 12% polyacrylamide gel (1.5 mm thick, 6 M urea, 40 watts). Size markers were obtained by end labeling (Alu I, Avr II, Bcl I, Bfa I, HindIII, or Nsi I) digestion of the appropriately radiolabeled bubble substrate (for restriction sites, see Fig. 1A) at 37°C for 1 (partial) or 3 h. Individual digests were mixed in various concentrations, heated to denature DNA products as above, and analyzed in parallel with exonuclease reactions. The gels were dried and visualized and quantitated by phosphorimaging analysis.

**Electrophoretic Mobility Shift Assay**—Fully duplex or bubble-containing substrate (32P-labeled, 0.5 fmol) was incubated with wild type WRN (12–72 fmol) or mock protein preparation (volume normalized to 30 min at 4°C in binding buffer (10 μl final volume) containing 20 μM HEPES-KOH (pH 8.0), 1 μM MgCl2, 0.1 mM EDTA, 100 μg/ml BSA, 0.5 mM dithiothreitol, 0.1% Nonidet P-40, 5% glycerol, 4% Ficoll, and 1 mM ATP-S or ATP. After addition of one-sixth volume of native dye (30% glycerol, 0.25% bromphenol blue, and 0.25% xylene cyanol), samples were separated by nondenaturing polyacrylamide (4%) gel electrophoresis. The DNA substrate containing a 21-nt bubble was used for this assay. DNA-protein complexes were visualized and quantitated by phosphorimaging analysis.

**DNA Unwinding Assay**—DNA substrates (21-nt bubble or full duplex, 32P-labeled on the 5′-end) 15–50 pmol each) were incubated for 15 min at 37°C with WRN-E84A (30–240 fmol) in WRN reaction buffer containing 40 μM Tris-HCl (pH 8.0), 4 μM MgCl2, 0.1 mM BSA, and 5 mM dithiothreitol. ATP (1 mM) was added unless otherwise indicated. The reactions were terminated by addition of 100 mM EDTA and 1.5 M NaCl to create a 30% hypotonic buffer and centrifuged. The DNA was dissolved in 1× TBE (1.5 mM NaCl, 0.1% xylene cyanol, and 0.015 units/tube T4 DNA ligase). The gels were stained with 0.5% xylene cyanol in TBE (1.5 mM NaCl) and visualized and quantitated by phosphorimaging analysis.

In this study, a defined DNA substrate containing an internal region of noncomplementarity was designed to closely approximate the localized melting of DNA duplexes that occurs during many metabolic processes. The DNA binding affinity and catalytic activities of recombinant wild type and mutant WRN proteins were examined on this substrate and compared with other model DNA substrates. We have demonstrated that, when compared with completely complementary substrates, a DNA substrate containing 21 unpaired nucleotides (nt) in both strands has extremely high affinity for the WRN protein. We have further characterized this binding and the effect of nucleotide cofactors by deoxyribonuclease I (DNase I) footprinting. We show that ATP hydrolysis is not required for high affinity binding of WRN to the melted region, and that the exonuclease domain does not bind stably to this substrate. Nevertheless, the WRN exonuclease function is highly active on this bubble substrate, and this activity is both independent of ATP binding or hydrolysis and further unwinding by WRN helicase function. Our results indicate coordination between the DNA binding and exonuclease domains of WRN and suggest that a non-complementary (heteroduplex) or melted region of DNA could be the physiological substrate of WRN. Such structures may be indicative of DNA replication, recombination, or repair intermediates formed in vivo that require processing by WRN helicase and/or exonuclease activity.
Unwinding of Bubble DNA—Enzymatic studies have demonstrated that WRN helicase requires a 3′ single-stranded region in the middle of a DNA substrate might satisfy the single-stranded DNA requirement of WRN helicase and might permit unwinding of an otherwise blunt-ended substrate to occur. We tested the ability of WRN to unwind our substrate containing an internal 21-nt bubble in comparison to fully complementary, blunt-ended double-stranded DNA. Because of the exonucleolytic activity of WRN wild type WRN protein on these substrates (see below), helicase assays were carried out with WRN-E84A mutant protein, which completely lacks exonuclease activity but retains unwinding capability. The WRN-E84A mutant was able to unwind the 80-mer substrate containing a 21-nt bubble at WRN concentrations of 60 fmol and above (Fig. 2A). Upon overexposure of gels, small amounts of slowly migrating forked structures were detected, corresponding to unwinding of a single duplex arm of the bubble substrate (data not shown). In contrast, WRN-E84A could not detectably unwind a completely complementary DNA substrate with blunt ends (Fig. 2C), in agreement with earlier reports (13, 24). The amount of helicase activity on the bubble substrate increased in a roughly proportional manner with increasing WRN concentration, whereas the fully duplex substrate was not unwound over the range of WRN concentration tested (Fig. 2D).

The unwinding activity on the bubble-containing substrate was inherent in the recombinant WRN-E84A, as mock-purified protein did not detectably unwind this substrate (Fig. 2B). As expected, WRN-catalyzed unwinding of the bubble substrate region in the middle of a DNA substrate might satisfy the single-stranded DNA requirement of WRN helicase and might permit unwinding of an otherwise blunt-ended substrate to occur.
requires ATP hydrolysis, as single-stranded products were not detectable in reactions lacking ATP (Fig. 2B) or containing ATPγS, a nonhydrolyzable ATP analog (data not shown). Furthermore, no unwinding activity was observed when reactions were incubated at 4°C. Thus, WRN could unwind our bubble-containing substrate to some degree, but, by manipulating reaction conditions, unwinding could also be prevented in order that binding and exonuclease activities on the intact substrate can be examined unambiguously.

Exonuclease Activity of WRN on Bubble DNA—In addition to its nucleic acid unwinding activity, WRN has also been demonstrated to possess an intrinsic 3’→5’ exonuclease activity (25–27). This activity is associated with the N-terminal region of the protein, and, in fact, deletion mutants containing only the N-terminal 308 amino acids retain exonuclease activity (26, 27). WRN exonuclease digests duplex substrates from a recessed 3’ end, but has little or no activity on fully complementary substrates with blunt ends (25, 28). However, a single-stranded region inside the duplex might overcome the need for a 5’ single-stranded overhang (recessed 3’ end) to achieve WRN exonuclease function. To test this possibility, we compared WRN exonuclease activity on our noncomplementary substrate containing a 21-nt bubble in the midst of a blunt-ended duplex (Fig. 1A) with a fully duplex substrate containing identical blunt ends (Fig. 1B). To prevent potential interference caused by WRN-catalyzed substrate unwinding, initial experiments were carried out in the absence of ATP. On the fully complementary DNA substrate, there was little or no degradation from the 3’ end of the labeled strand (Fig. 3C), in agreement with previous reports (25, 28). In contrast, a robust 3’→5’ exonuclease activity was evident from the end 24 bp from the beginning of the bubble on the noncomplementary substrate, even in the absence of ATP (Fig. 3A). A similarly high level of activity was also observed when the other strand of the substrate was labeled, where the detectable degradation started 35 bp from the edge of the bubble (Fig. 3B). By comparison, WRN exonuclease activity on a duplex substrate with a recessed 3’ end but without a bubble was barely detectable only at concentrations of ~150 fmol and increased progressively from that point over the testable range of WRN concentration (Fig. 3D).

When WRN-E84A protein containing a point mutation in the conserved exonuclease domain was incubated with the bubble-containing substrate, no degradation was observed (Fig. 3E), confirming that the avid exonuclease activity associated with the wild type protein is inherent to WRN. The susceptibility of the bubble-containing and recessed end substrates to WRN exonuclease
was assessed by comparing the percentages of undigested substrate remaining (compared with an untreated control) after incubation with various concentrations of wild type WRN. These measurements indicate that, in the absence of ATP, the “preferred” duplex substrate containing a 3’ recessed end (Fig. 3D) required about 100 times more WRN enzyme than the bubble-containing substrate (Fig. 3A and B) to achieve an ~50% reduction of the amount of original, undigested substrate. Thus, exonuclease activity was initiated much more readily on the bubble-containing substrate when compared with the 3’ recessed end substrate. Another obvious difference in comparing the degradation patterns of the 3’ recessed end substrate with that of the bubble substrate is the extent of inward digestion. Only a few nucleotides were removed from the 3’ end of the recessed end substrate (Fig. 3D), suggesting a distributive mode of exonuclease activity. In contrast, WRN

![Image of Fig. 3](http://www.jbc.org/)

**Fig. 3. Highly efficient 3’ → 5’ exonuclease activity of WRN on bubble substrate.** A, WRN exonuclease degradation of the G-rich strand of the bubble substrate (0.5 fmol, 32P-labeled on the G-strand). B, WRN exonuclease degradation of the C-rich strand of the bubble substrate (0.5 fmol, 32P-labeled on the C-rich strand). C, WRN exonuclease treatment of the fully duplex (see Fig. 1B) control substrate (0.5 fmol, 32P-labeled on C80 oligomer). D, WRN exonuclease treatment of 3’ recessed end substrate (51-mer, 32P-labeled, annealed to 57-mer, ~0.5 fmol). E, WRN-E84A treatment of the bubble substrate, as in B. All reactions were incubated with WRN (wild type, 2.25–300 fmol, or WRN-E84A, 120–240 fmol, as indicated) for 1 h at 37 °C in the absence of ATP. After strand separation was achieved by heating at 90 °C, the DNA products were resolved by denaturing polyacrylamide (14%) gel electrophoresis and then radioactive species were visualized by phosphorimaging. Arrows denote the point of attack for the 3’ → 5’ exonuclease activity of WRN. The positions of nucleotide markers and the bubble region (curved brackets) were obtained by running denatured restriction digests (see Fig. 1A for sites) of G-rich strand-labeled (panel A; HindIII, 16 nt; AluI-partial, 18 nt; BfaI-partial, 24 nt; NspI, 66 nt; BclI, 73 nt) or C-rich strand-labeled (panel B; NspI, 18 nt; AluI, 62 and 23 nt; BfaI, 54 nt; HindIII, 60 nt; AvrII, 71 nt) substrate alongside exonuclease digestions. The location of the 62-nt AluI marker has been omitted to avoid crowding.
The heightened exonuclease activity on the substrate containing the noncomplementary region (in comparison with the fully duplex and 3′ recessed end substrates) could be attributable to properties of the exonuclease domain alone, or to the coordination of the exonuclease domain with an affinity of some other part of the WRN protein to the melted region of the duplex. This question was addressed by examining the activity of WRNΔ369–1432, a mutant WRN protein containing only the N-terminal exonuclease domain, on the bubble-containing substrate. This truncated form of WRN is an active 3′ → 5′ exonuclease on DNA substrates with 3′ recessed ends (Ref. 27 and Fig. 4). Notably, this level of WRNΔ369–1432 exonuclease activity on the 3′ recessed end substrate is comparable with what is observed using wild type WRN (compare Figs. 3D and 4). However, when WRNΔ369–1432 was incubated with either the bubble containing or the fully duplex substrate, no exonuclease activity was detected (Fig. 4). These results suggest that another part of WRN outside of the N-terminal 368 amino acids is required to achieve the elevated exonuclease activity on the 3′ blunt ends of the bubble substrate. Thus, it is likely that regions within the helicase domain and/or the C-terminal portion of WRN have significant affinity for a melted region of duplex (and/or the associated junctions between single- and double-stranded DNA), which mediates the potent 3′ → 5′ exonuclease activity at distances up to 35 bp away.

WRN exonuclease activity on duplex DNA substrates containing recessed 3′ ends has been shown to be stimulated dramatically by the presence of ATP (28). We wanted to examine whether the potent exonuclease activity observed on the 3′ ends of the bubble-containing substrate was likewise affected by binding or hydrolysis of an ATP cofactor. To this end, we incubated the bubble substrate with wild type WRN in reaction mixtures containing ATP, nonhydrolyzable analog ATPγS, or lacking nucleotide altogether. Surprisingly, the substrate was digested extensively by the 3′ → 5′ exonuclease activity of WRN under all three conditions (Fig. 5A). The amount of intact substrate remaining was similar (~4%) in the presence or absence of ATP, and slightly higher (~10%) in the presence of ATPγS. In addition, differences in digestion pattern were noticeable when ATP, ATPγS, and minus ATP reactions were compared. Exonuclease activity in the presence of ATP increased along with WRN concentration, while remaining relatively constant in the presence of ATPγS or in the absence of ATP over the same 5-fold range of WRN concentration (Fig. 5A). These results were supported by similar experiments using the WRN-K577M protein, which lacks ATP hydrolysis and DNA unwinding activity. In the absence of ATP or in the presence of ATP or ATPγS, the exonuclease activities and patterns of WRN-K577M on the bubble substrate are extremely
similar to one another (Fig. 5B). Thus, two lines of evidence demonstrate that ATP hydrolysis is not required for and does not greatly affect the ability of WRN exonuclease to both initiate and continue degradation on the 3′ ends of our substrate containing a 21-nt bubble. Our results are in contrast with an earlier report (28) that found ATP-dependent stimulation of WRN exonuclease activity on duplex substrates with recessed 3′ ends, but perhaps more significantly also differ from those of a recent report (35) that indicated an ATP hydrolysis requirement in the 3′ exonucleaseic degradation by WRN of a variety of substrates containing smaller bubbles or loops. Nevertheless, our data clearly show that ATP binding and hydrolysis are not required for exonucleaseic degradation of the 3′ ends of a DNA substrate containing a 21-nt noncomplementary region in the midst of duplex DNA.

**High Affinity Binding of WRN to Bubble DNA**—Studies of WRN helicase and exonuclease functions on various types of DNA substrates suggest that WRN may act preferably on alternate (nonduplex) DNA structures (16, 24, 35). It is likely that this preference is manifested through differences in binding of WRN to specific structural features of DNA substrates. We used the EMSA to determine whether WRN could form a stable complex with a DNA substrate containing a noncomplementary bubble. To prevent WRN helicase and exonuclease activities on DNA substrates, both the incubation and electrophoretic steps of this assay were carried out at 4 °C. By this analysis, WRN formed stable complexes with the bubble-containing substrate in the presence of ATPγS (Fig. 6) or to a slightly lesser extent in the presence of ATP or without nucleotide cofactors (data not shown). By contrast, no protein-DNA complexes were detected when WRN was incubated with fully duplex DNA or when mock protein preparations (lacking recombinant WRN) were incubated with either DNA substrate (Fig. 6 and data not shown). Thus, WRN formed stable complexes with DNA substrates containing a 21-nt noncomplementary region.

**DNase I Footprint of WRN on Bubble DNA**—Although the above analysis identifies stable complexes between WRN and DNA containing a noncomplementary bubble, a more precise examination of these complexes requires a more sensitive method. DNase I footprinting has been widely used to pinpoint the binding sites of proteins on specific DNA sequences and structures. Notably, this method is also an equilibrium binding technique, and thus more sensitive than nonequilibrium techniques such as EMSA and filter binding assays. We employed this method to determine whether WRN binds stably to a DNA substrate containing a 21-nucleotide noncomplementary region, and to map the regions of DNA covered by bound protein (as measured by inhibition of DNase I endonuclease activity).

As for the earlier EMSA experiments, WRN binding and DNase I endonuclease inhibitions were typically carried out at 4 °C in ATP or ATPγS to prevent WRN unwinding and exonuclease activities and thus maintain integrity of the DNA substrate structure. WRN unwinding activity was undetectable at 4 °C (data not shown), and the lack of exonuclease activity by WRN at 4 °C was demonstrated by the incubation of WRN and DNA substrate without subsequent DNase I treatment (Fig. 7, A–D, second lane from left). DNase I digestion of the substrate containing a 21-nucleotide bubble surrounded by 35-bp (long arm) and 24-bp (short arm) duplex regions is shown in Fig. 7 (B and D, third lane from right). In general, the DNase I digestion was weak in the noncomplementary region, probably because of its single-stranded character. Nevertheless, increasing concentrations of WRN clearly inhibited DNase I incision in the noncomplementary region of the substrate (Fig. 7, B and D, lanes 3–6). WRN binding to this substrate was complete by 5 min (data not shown) and protected a region surrounding the noncomplementary region, being particularly evident to the short (24 bp) arm of the substrate on the C-rich strand (Fig. 7B). Using relevant restriction sites present in the substrate (Fig. 4A), we estimate that, on the C-rich strand, WRN protects a region that includes the entire bubble, 10 nt of the short arm, and 5–6 nt of the long arm. In addition, the boundary between the region bound by WRN and unbound areas of the substrate could be inferred by the appearance of a significant DNase I-hypersensitive site (indicated by a thick arrow) at −7 nt as well as a minor hypersensitive site (indicated by a thin arrow) at 13 nt from the edge of the bubble toward the 3′ end of the C-rich strand on the long arm (Fig. 7B). Although the pattern is not as clear because of greater inhibition of DNase I incision on the G-rich strand in the noncomplementary region, WRN binding again inhibited DNase I incision in the vicinity of the bubble (Fig. 7D), covering the entire noncomplementary region plus −8 and 6 nt on the 35- and 24-bp arms, respectively, and creating DNase I-hypersensitive sites on the short arm (toward the 3′ end of the G-strand). Thus, the binding of WRN to substrate appears to encompass the noncomplementary region with a slight asymmetry toward the 5′ end of each strand. The
binding affinity of WRN for this substrate was calculated from the concentration at which ~50% of the intensity of a specific band (indicated by asterisk) had disappeared to give a $K_a$ of 4 × 10^6 M⁻¹. By comparison, no indication of DNase I inhibition was observed on either strand of the fully duplex control substrate, even using WRN concentrations that were greater than 4-fold higher than necessary to yield a complete footprint on the bubble substrate (Fig. 7, A and C). On the bubble-containing substrate, similar footprinting patterns were observed when an exonuclease-deficient/helicase-proficient mutant was used instead of wild type protein. This is the case even when WRN bound with high affinity to a noncomplementary region of DNA under noncatalytic (4°C) and catalytic (37°C) conditions.

In contrast, the WRNΔ369-1432 deletion mutant protein containing only the exonuclease domain did not create a detectable footprint (even at significantly higher molar ratios of protein to DNA) in the vicinity of the bubble (Fig. 9). The absence of a footprint near the bubble with WRNΔ369-1432 indicates that this region of the protein is not sufficient (and probably not involved) for the binding observed with the full-length protein. Notably, no inhibition of DNase I incision was observed near either end of the bubble-containing substrate using wild type WRN, WRN-K477M, WRN-E84A, or WRNΔ369-1432 when the assay was carried out at 4°C (Figs. 7B and D, 9, and 10B).

When DNase I assay was done with WRN-E84A at 37°C (Fig. 8). These results suggest that, although the exonuclease activity was directed at both ends of the substrate (see Fig. 3, A and B), the interaction of the WRN exonuclease domain with the DNA ends must be such that DNase I incision in those regions was not affected.

We also tested the effect of nucleotide cofactors on the binding of WRN to the bubble containing substrate. As ATP hydrolysis is prevented by carrying out the binding and footprinting incubations at 4°C, no difference was observed when WRN was incubated with the bubble substrate in the presence of ATP or ATPγS (data not shown). In contrast, when nucleotide cofactors were omitted during the binding incubation, the footprint was much less distinct at comparable WRN concentrations (Fig. 10A). At a 2–4-fold higher WRN concentration in the absence of ATP, the pattern of DNase I digestion was similar if not identical to the pattern observed in the presence of ATPγS (Fig. 10A). The similarity between these footprints indicates that the noncomplementary region is bound by WRN in the same conformation whether nucleotide cofactor is associated with the protein or not. To further test this hypothesis, we compared the binding of the wild type protein with that of WRN-K577M containing a point mutation in a conserved nucleotide binding/hydrolysis motif that eliminates ATP hydrolysis and helicase activity (11, 12). As expected, WRN-K577M had no unwinding activity on this bubble substrate (data not shown). When WRN-K577M was incubated with the bubble substrate in the presence of ATPγS, it protected an area surrounding the bubble on the C-rich strand WRN from DNase I digestion (Fig. 10B).
pattern similar to that for wild type WRN. However, when compared with wild type protein in the presence of ATP-γ-S (1 mM), a significantly higher concentration of WRN-K577M was necessary to achieve the same level of protection, as was the case with the wild type WRN footprint in the absence of ATP (Fig. 10A). Taken together, our results indicate that the binding of WRN to the bubble-containing substrate does not require a nucleotide cofactor. However, nucleotide binding significantly enhances the binding of WRN to the noncomplementary region of the substrate. By extension, it also appears that mutation of the conserved lysine to methionine in motif I of the RecQ helicase domain may eliminate not only the ATP hydrolysis activity but also the ATP binding capability of WRN. Another possibility is that this mutation lowers the DNA binding affinity of the WRN-K577M protein. However, the manner in which full-length WRN binds to the bubble structure appears to be similar, regardless of the presence or absence of nucleotide cofactors. Most importantly, we can conclude that the highly specific binding of WRN to the melted region of our substrate (that is observed with or without ATP) underlies the potent 3′ → 5′ exonuclease activity that occurs on the nearby blunt ends.

**DISCUSSION**

Genomic instability in the premature aging and cancer-prone hereditary disease, WS, is thought to be caused by DNA metabolic defects that result from a lack of WRN protein. This hypothesis has been strengthened by the identification and characterization of 3′ → 5′ helicase and 3′ → 5′ exonuclease activities of WRN. In this study, a substrate containing a 21-nt noncomplementary region (bubble) in the midst of duplex DNA (Fig. 1A) has been shown to be bound with high affinity by WRN using the EMSA and DNase I protection (footprinting) assays. The footprinting technique was further exploited to determine the precise region of DNA bound by WRN and the participation of the exonuclease and nucleotide binding domains of WRN in binding to this structure. We also demonstrate that this high affinity binding underlies an extremely efficient exonuclease activity directed at the nearby 3′ ends of this substrate. In addition, a significant percentage of this substrate can be completely unwound by WRN helicase activity, although only at substantially higher WRN concentrations than necessary to achieve optimal exonuclease activity. We hypothesize that WRN binding to a melted region of duplex, as demonstrated by DNase I protection, serves as an anchor that permits the exonuclease domain of WRN to access nearby 3′ ends at a much higher frequency than would occur by constant cycling of WRN on and off of a substrate. This notion would explain the vastly increased amounts of initiation and inward degradation of WRN exonuclease activity on this substrate containing a 21-nt noncomplementary region, in comparison to fully duplex and 3′ recessed end substrates.

The DNase I assays reveal several interesting aspects regarding WRN protein binding to the bubble-containing DNA substrate. ATP is not required for stable binding of WRN to the bubble region, although ATP binding apparently increases the affinity of WRN for substrate significantly. In contrast, a completely duplex substrate of equal length and similar sequence was not detectably bound by WRN, even at substantially higher protein concentrations. From these experiments, we calculate the Kₐ for WRN binding in the presence of ATP (or ATP-γ-S) to the bubble substrate to be 4 × 10⁸ M⁻¹. By comparison, the Kₐ for a long single-stranded DNA substrate was estimated to be 10⁶ to 10⁷ M⁻¹ and at least 5-fold higher than for double-stranded DNA (29). Thus, WRN binds to the bubble region (or the junctions between double- and single-stranded DNA contained therein) with affinity comparable with that for
single-stranded DNA and certainly much better than a linear double-stranded DNA substrate. WRN binding to the bubble-containing substrate protects an area from DNase I digestion that includes the entire melted region of 21 unpaired nucleotides on each strand as well as limited sequence (7–10 bp) within the duplex regions on either side. Therefore, WRN protects an area of DNA on each strand 35–40 nt in length with a slight asymmetry toward the 5’ side of the bubble. Interestingly, there is no detectable area of protection in the vicinity of either end of this substrate, suggesting that 3’ end binding events that occur during exonucleolytic processing are transient, and potentially pointing toward a distributive mechanism for WRN exonuclease function. However, we cannot rule out the possibility that stable binding of the exonuclease domain to the 3’ end does occur under catalytic conditions (using exonuclease-proficient WRN at 37 °C) that cannot be tested because of WRN-associated digestion of DNA.

Our bubble-containing substrate is an excellent substrate for the 3’→5’ exonuclease activity of WRN. WRN exonuclease can act at either blunt end, degrading from the 3’ end back toward and, with some frequency, through and beyond the noncomplementary region. By comparison, the comparable blunt-ended fully duplex substrate and a substrate with a 3’ recessed end are poor substrates for WRN exonuclease activity. This indicates that high affinity binding to the bubble region is possible for the enhanced exonuclease activity initiated at the blunt ends 35 and 24 bp away. The WRNΔ369–1432 mutant containing only the intact exonuclease domain cannot bind to or digest the bubble-containing substrate, indicating that the elevated exonuclease activity of wild type WRN requires portions of the protein outside of the exonuclease domain to bind to the melted region.

Earlier studies have demonstrated that the presence of an ATP cofactor can stimulate the exonuclease activity of WRN on duplex substrates with 3’ recessed ends (28). Our results using substrate containing a 21-nt bubble show that the level of WRN exonuclease activity is very high regardless of the presence or absence of nucleotide cofactor. The exonuclease activity of the ATPase/helicase-deficient WRN-K577M protein on this substrate is strikingly similar to that of wild type WRN in the absence of ATP, consistent with the idea that ATP binding and hydrolysis are not necessary for optimal degradation of this particular bubble substrate. The different patterns of WRN exonuclease digestion observed with or without ATP cofactors could be attributed either to ATP-influenced association and dissociation dynamics of WRN to the substrate, or to alteration, denaturation, or ATP-mediated unwinding of the DNA substrate as digestion proceeds. Although ATP binding and hydrolysis are dispensable for the heightened exonuclease activity of WRN on the ends of our bubble substrate, certainly ATP binding and hydrolysis play a role in substrate binding and the dynamics of exonuclease activity on several types of DNA structures (Refs. 28 and 35, and this study).

A discussion of the results of this study would not be complete without comparison to a recently published report (35) that examined WRN exonuclease activity on several types of DNA structures including small bubbles and extrahelical single-stranded loops. Both studies show that an internal bubble permits WRN 3’→5’ exonuclease activity on a nearby blunt end duplex, a structure that is resistant to digestion in the
context of fully complementary DNA. However, WRN exonuclease activity on a 46-bp substrate with an 8-nt bubble appears to require ATP hydrolysis and is strongly inhibited at 5 nt from the junction of the bubble (or loop) with duplex DNA (35). In contrast, our results using an 80-bp substrate with an internal bubble of 21 nt indicate that (i) WRN exonuclease activity occurs optimally even without ATP or helicase activity and (ii) digestion occurs up to, through, and to some extent beyond the bubble structure without substantial inhibition. The most likely explanation for these differences could stem from the larger size of our substrate and bubble (80 bp, 21-nt bubble) in comparison to theirs (46 bp, 8-nt bubble). Their results suggest that an 8-nt bubble may have to be further unwound by WRN helicase (with the concomitant requirement for ATPase activity) to create a stable binding site, whereas our results demonstrate that a 21-nt bubble constitutes a stable binding site for WRN without additional unwinding. The inhibition of exonuclease activity at 5 nt from the edge of the bubble could also be a result of the size of their substrate and its ATPase/helicase requirement. The unwinding activity that occurs in the presence of ATP combined with exonuclease activity at both ends would conceivably cause the short substrate to fall apart as digestion proceeds. As single-stranded DNA is not digested by WRN exonuclease, the denatured substrate is resistant to further degradation. As our substrate is significantly longer and subject to exonuclease degradation in the absence of unwinding activity, it probably retains double-stranded character even after significant degradation from both ends, thus facilitating a higher extent of digestion up to and through the bubble region. Another possibility would be that, under certain circumstances, binding of the WRN helicase domain to the bubble may simply physically inhibit the extent of degradation by the exonuclease domain.

WRN is unique among the human RecQ members in that it

**FIG. 11.** Coordinated action of the bipartite and bifunctional WRN protein. Full-length WRN protein (shaded) is depicted as containing linked but physically separate helicase (hel, circular) and exonuclease (exo, oval) domains. Proposed directions of movement of individual domains are indicated with dashed arrows. A, uncoupled helicase and exonuclease activities of WRN on 3' overhang and 3' recessed ends, respectively, of a duplex substrate. B, coordinated activity of WRN on partially unwound duplex DNA substrates. Top, as demonstrated in this study, binding of WRN to unwound or noncomplementary region of DNA (probably mediated by the helicase domain) facilitates the activity of the 3' → 5' exonuclease domain at a nearby blunt end, leading to degradation of one strand toward the melted region. Bottom, similarly, the binding of WRN to unwind duplex can direct the exonuclease activity to a nick, as shown for DNA containing an extrahelical loop by Shen and Loeb (35), leading to 3' → 5' degradation of one strand toward the melted region from that discontinuity in double-stranded DNA. C, dynamic models for coordinated action of the helicase and exonuclease domains of WRN. Left, high affinity binding to an unwound region of DNA facilitates exonuclease activity at a more distant end or nick, accomplished either by bending or spooling of flexible DNA, or by helicase-mediated movement of the bubble along DNA toward the discontinuity. Right, also possible is the concerted degradation of long stretches of one strand by helicase-mediated movement of the bubble ahead of the tethered exonuclease domain.
contains 3' → 5' exonuclease activity as well as the requisite 3' → 5' helicase activity. In this report, we have demonstrated that a helicase-deficient WRN protein is active as an exonuclease and vice versa, demonstrating that the exonuclease and helicase activities can occur independently of one another. Moreover, the WRNΔ369–1432 mutant containing only the exonuclease domain is catalytically active (Ref. 27 and this study), indicating that the N-terminal part of WRN folds into a functional domain. This evidence suggests that wild type WRN is bipartite as well as bifunctional. Early experiments (13, 25, 28) implied that the domains have opposing specificities; helicase function requires a single-stranded region 3' to the duplex to be unwound, and exonuclease function requires a single-stranded region 5' to the duplex to be degraded. This concept is clarified in Fig. 11A, which depicts the individual catalytic activities of WRN acting at the opposite ends of a DNA duplex with flanking 3' and 5' single-stranded regions. Our experiments suggest another scenario; WRN binds to a partially melted region of DNA and can then degrade one strand of the DNA from certainly a nearby end or nick (Fig. 11B) and possibly a more distant end or nick through a DNA bending or threading mechanism (Fig. 11C, left). WRN helicase activity could increase the original size of the unwound region to create a more stable binding site and/or move the bubble along DNA either toward or away from the end or nick being acted on by the exonuclease domain. Coordinated movement of the WRN helicase and exonuclease domains along one strand of a duplex in a 3' → 5' direction could potentially allow the degradation of long stretches of that same strand (Fig. 11C, right). This kind of mechanism might be very useful in a number of DNA metabolic pathways.

The extreme affinity and activity of WRN helicase/exonuclease on DNA substrates containing noncomplementary (or unwound) regions is likely to reflect the physiological function of WRN in DNA metabolism. WRN can unwind DNA structures provided the existence of a 3' single-stranded region, but has not yet been shown to be able to unwind DNA that is in a fully duplex form. However, several situations can be envisaged by which WRN might function to bind and at least partially unwind regions within essentially continuous (chromosomal) DNA: (i) binding/unwinding by WRN at specific sequences or sites, (ii) participation of WRN in a complex that initiates or assists in duplex melting, (iii) attraction of WRN to a "bubble" structure already partially unwound by activities of other proteins/complexes, and (iv) specific binding to noncomplementary or unwound regions of DNA that occur during or as a result of DNA metabolic processes. Evidence implicates WRN function at regions destined to become or already unwound. FFA-1, the WRN ortholog in S. cerevisiae, appears to participate with RPA at replication foci (30). In agreement with a possible role for WRN at replication origins and/or forks, WS cells have reduced rates of replication initiation and elongation (31, 32), likely resulting in the observed extension of S phase (33). Both WRN and its S. cerevisiae homolog, Sgs1, have been proposed to have positive transcriptional roles (34, 36), perhaps mediated through assistance in creation, expansion, or movement of transcription bubbles.

In contrast to the other human RecQ homologs, any proposed role for WRN should consider its combined helicase and exonuclease activities. Thus, WRN might create an optimum binding site through either its unwinding activity or its ability to bind to already melted regions of duplex DNA, then act coordinately as an exonuclease at a nearby 3' end, as demonstrated by our in vitro experiments. A significant body of evidence suggests that the RecQ family proteins may be involved in recombination or anti-recombinogenic pathways (1). One possibility suggested by our experiments is that WRN helicase and exonuclease could act in concert to degrade heteroduplex DNA formed during nonhomologous (illegitimate) recombination. Alternatively, as strand invasion into duplex DNA is an initiating step in recombination, WRN or its RecQ cousins may be involved in assisting the strand invasion step or conversely, disrupting the D-loop structure created during strand invasion. Recently, BLM has been shown to bind and melt D-loops (37). Through its analogous helicase activity, WRN might also disrupt strand invasion intermediates and perhaps even degrade the invading 3' strand via its associated exonuclease activity. In support of a potential role in recombination or an anti-recombinogenic pathway, WRN has been shown to migrate Holliday junctions in vitro (17). It has also been suggested that through an interaction with the Ku heterodimer, WRN may be involved in end processing during nonhomologous end joining pathway of DNA double-strand break repair (38–40). Perhaps in coordination with Ku, a complex that may also have some DNA unwinding capability, bubbles or forked intermediates are created at damaged ends that are subsequently acted on by WRN exonuclease. Importantly, when provided a small extra-helical loop in the midst of duplex DNA, WRN exonuclease has been shown to degrade at a nearby nick 3' to the bubble (35). Such an activity is reminiscent of exonucleases that act from nicks created on the newly replicated strand during the mismatch repair process. A defect in this exonucleolytic processing might be expected to increase the persistence of these nicks and perhaps lead to double-strand breaks and a concomitant increase in deletion, insertion, and translocation mutations that would fit with some aspects of the WS genomic instability phenotype. A mismatch repair defect has been reported in WS cells (41), but further investigations in this area are needed. Another possible role for WRN could be the processing of Okazaki fragments that occur as a result of lagging strand replication, although at this time no direct evidence implicates WRN in such a pathway. Examination of WRN activities on DNA structures that more closely reflect true physiological substrates and a rigorous analysis of the DNA metabolic deficiencies of WS cells should lead to elucidation of the exact function of the WRN protein.

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