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Characterization of Werner syndrome protein DNA helicase activity: directionality, substrate dependence and stimulation by replication protein A

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ABSTRACT

Werner syndrome is an inherited disease characterized by premature aging, genetic instability and a high incidence of cancer. The wild type Werner syndrome protein (WRN) has been demonstrated to exhibit DNA helicase activity *in vitro*. Here we report further biochemical characterization of the WRN helicase. The enzyme unwinds double-stranded DNA, translocating 3′→5′ on the enzyme-bound strand. Hydrolysis of dATP or ATP, and to a lesser extent hydrolysis of dCTP or CTP, supports WRN-catalyzed strand-displacement. K_m values for ATP and dATP are 51 and 119 μM, respectively, and 2.1 and 3.9 mM for CTP and dCTP, respectively. Strand-displacement activity of WRN is stimulated by single-stranded DNA-binding proteins (SSBs). Among the SSBs from *Escherichia coli*, bacteriophage T4 and human, stimulation by human SSB (human replication protein A, hRPA) is the most extensive and occurs with a stoichiometry which suggests direct interaction with WRN. A deficit in the interaction of WRN with hRPA may be associated with deletion mutations that occur at elevated frequency in Werner syndrome cells.

INTRODUCTION

Werner syndrome (WS) is an autosomal recessive genetic disorder characterized by aging in early adulthood (1–3). Individuals with WS frequently develop age-related diseases prematurely including atherosclerosis, osteoporosis, type II diabetes mellitus, cataracts and a variety of unusual malignant neoplasms. WS cells also exhibit an aging phenotype in culture, characterized by a reduced replicative life-span (4) and alterations in DNA synthesis (5–7). In addition, WS cells exhibit genetic instability, manifested as variegated translocation mosaicism (8) and increased mutation rates (9). Interestingly, the mutations obtained in WS cells in culture are predominantly large deletions (9).

The gene defective in WS has been localized to chromosome 8 at 8p12 (10), and its cDNA has been cloned and demonstrated to encode a 1432 amino-acid protein (11). The WS gene product, WRN, was predicted to function as a DNA helicase on the basis of homology with the *Escherichia coli* RecQ family of helicases. Recently, the WRN protein was expressed in a recombinant baculovirus system and demonstrated to exhibit DNA helicase activity (12,13). *Escherichia coli* RecQ is a 3′→5′ DNA helicase involved in the RecF pathway of homologous recombination (14). It has been shown that *RecQ* is required to suppress illegitimate recombination between λ phages, and thus to function in the maintenance of genetic stability (15). The RecQ helicase may also participate in the resumption of DNA replication at the replication fork following encounter with a UV-induced lesion (16,17). Another *RecQ* family member, the Bloom's syndrome (BS) gene, is mutated in a human genetic disorder characterized by cancer predisposition and genomic instability; the BS cDNA has also been cloned and the protein product demonstrated to exhibit DNA helicase activity *in vitro* (18,19). Similarly, *SGS1* in *Saccharomyces cerevisiae* (20,21) and *RQH1* in *Schizosaccharomyces pombe* (22,23) are *RecQ* homologs and are required for the maintenance of genetic stability. Sgs1 has been demonstrated to exhibit DNA helicase activity *in vitro* (24) and may provide a model system to evaluate the function of the *WRN* gene product.

Mutations in the *WRN* gene found in WS patients are mostly stop codons or exon deletions that result in premature termination of translation (11,25–27). Interestingly, no missense mutation has been observed in the *WRN* gene. Study of the nuclear localization of wild type and mutant WRN proteins revealed a nuclear localization signal in the C-terminal region of the wild type protein that is deleted in most WS patients (28). Lack of WRN helicase activity in the nucleus may therefore account for the phenotype of WS cells.

In order to understand the role of WRN helicase in the maintenance of genomic integrity, we have expressed the WRN protein in a baculovirus expression system and demonstrated its DNA helicase activity (12). Here we report that its direction of translocation is 3′→5′, and that it can utilize a variety of nucleoside triphosphates, i.e. ATP, dATP, CTP and dCTP, as an energy source for translocation. We also report that WRN helicase activity is specifically enhanced by human single-stranded DNA-binding protein.

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MATERIALS AND METHODS

Nucleoside triphosphates

dNTPs were obtained from Perkin Elmer, and NTPs were purchased from Pharmacia Biotech. Radioactive [γ-^{32}P]ATP and [α-^{32}P]dCTP were obtained from New England Nuclear.

Oligonucleotides and DNA

Chemically synthesized, HPLC purified oligonucleotides were obtained from Operon Technologies Inc. The 42mer (5′-TAGCATGTCAATCATATGTACCCCGGTTGATAATCAGAAAAG-3′) is complementary to nucleotides 6768–6809 of M13mp2 (+) strand DNA. The 34mer (5′-TAGCATGTCAATCATATGTACCCCGGTTGATAAT-3′) is complementary to nucleotides 6768–6801 of M13mp2 (+) strand DNA. The 46mer (5′-GCGCGGAAGCTTGGCTGCAGAATATTGCTAGCGGGAATTCGGCGCG-3′) is a random sequence oligonucleotide; the 20mer (5′-CGCTAGCAATATTCTGCAGC-3′) is complementary to the central region while the 23mers (5′-CGCGCCGAATTCCCGCTAGCAAT-3′) and (5′-ATTCTGCAGCCAAGCTTCCGCGC-3′) are complementary to the 3′- and 5′-segments, respectively. Bacteriophage M13mp2 (+) strand DNA was purified by a standard method as described (29).

Enzymes

Recombinant WRN with a hexa-histidine tag at the N-terminus was expressed in Sf9 insect cells and purified by Ni^{2+}-chelation chromatography, as described (12). The purified protein is ~90% homogeneous as visualized on the Coomassie blue-stained SDS polyacrylamide gel. The protein concentration was determined by the Bio-Rad protein assay kit using BSA as a standard. Homogeneous *E.coli* helicase II (UvrD) was a gracious gift from Dr Lawrence Grossman (The Johns Hopkins University). Recombinant human RPA containing all three subunits (RPA70, RPA32 and RPA14), purified from *E.coli* simultaneously expressing the three hRPA genes, was a generous gift from Dr Marc S.Wold (University of Iowa). *Escherichia coli* SSB was purchased from Pharmacia Biotech, and T4 gene 32 protein was from Boehringer Mannheim. DNA labeling enzymes, T4 polynucleotide kinase for 5′-end ^{32}P-labeling and Klenow fragment (3′→5′ exo$^-$) for 3′-end [α-^{32}P]dNTP incorporation, were purchased from New England BioLabs. The restriction enzyme *Rsa*I was also obtained from New England BioLabs.

DNA helicase substrates

The DNA substrate for determining the dependence of DNA unwinding on NTP and dNTP was prepared by incubating the 5′-^{32}P-labeled 20mer and the 46mer in 1:3 molar ratio in annealing buffer (10 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$). Annealing was carried out by placing the reaction mixture in a boiling water bath (800 ml) for 3 min and then letting it cool gradually to room temperature. The longer partial duplex substrate for determining the stimulatory effects of SSBs on WRN helicase was prepared by hybridizing the 5′-^{32}P-labeled 42mer with single-stranded (ss) M13mp2 DNA at a molar ratio of 2:3. Substrates for determining the directionality of WRN helicase were prepared in three ways. The first partial duplex substrate, which contains a 19mer complementary to the 5′-end and a 34mer complementary to the 3′-end of a linear ssM13mp2 DNA, both in a blunt-ended manner, was prepared sequentially as follows. The 5′-^{32}P-labeled 42mer was hybridized to circular ssM13mp2 DNA as described above. The annealed 42mer was extended by the Klenow fragment (exo$^-$) in the presence of dATP and [α-^{32}P]dCTP to yield a 53mer containing five radioactive dCMPs at the 3′-end. This 53mer/ssM13mp2 DNA partial duplex was then digested with restriction enzyme *Rsa*I to yield a blunt-ended, partially-duplex, linear ssM13mp2 DNA product which contains a 5′-^{32}P-labeled 34mer complementary to its 5′-end and a 3′-[α-^{32}P]dCMP-labeled 19mer complementary to its 3′-end. The second directionality substrate was prepared similarly, by first hybridizing a 5′-^{32}P-labeled 34mer with circular ssM13mp2 DNA, and then extending one nucleotide at the 3′-end with [α-^{32}P]dCTP and Klenow fragment (exo$^-$). The resulting 35mer/ssM13mp2 DNA partial duplex was then digested with *Rsa*I to yield a linear M13mp2 DNA containing partial duplex at each blunt-ended terminus, with a 5′-^{32}P-labeled 19mer located at the 5′-terminus and a 3′-[α-^{32}P]dCMP-labeled 16mer at the 3′-terminus. The third directionality substrate is a pair of blunt-ended partial-duplexes with a single complementary oligonucleotide residing on one or the other end. Two 5′-^{32}P-labeled 23mers, each complementary to one half of a 46mer template, were hybridized to the 46mer as described before, in separate reactions containing one of the 23mers and the 46mer in a molar ratio of 1:3. This produced a pair of directionality substrates with a blunt-ended partial duplex at the 5′-end or the 3′-end.

DNA helicase assay

DNA helicase activity was measured in reaction mixtures (10 µl) containing ^{32}P-labeled DNA substrate (0.1 pmol for oligonucleotide substrates and 1 fmol for ssM13mp2 partial duplex substrates), 1 mM ATP (or the indicated concentrations of NTP or dNTP), and SSB when indicated, in 40 mM Tris–HCl (pH 7.4), 4 mM $MgCl_2$, 5 mM DTT, 100 µg/ml BSA. Reactions were terminated by adding 2 µl of 40% glycerol, 50 mM EDTA, 2% SDS, 3% bromophenol blue and 3% xylene cyanol. Partial duplex substrate and displaced single-stranded oligonucleotide product were resolved by electrophoresis at 4°C for 2 h at 300 V (20 V/cm) through a 12% polyacrylamide gel in 1× TBE (90 mM Tris base, 90 mM boric acid, 1 mM EDTA) and visualized by autoradiography or quantitated by PhosphorImager (Molecular Dynamics) analysis of the dried gels.

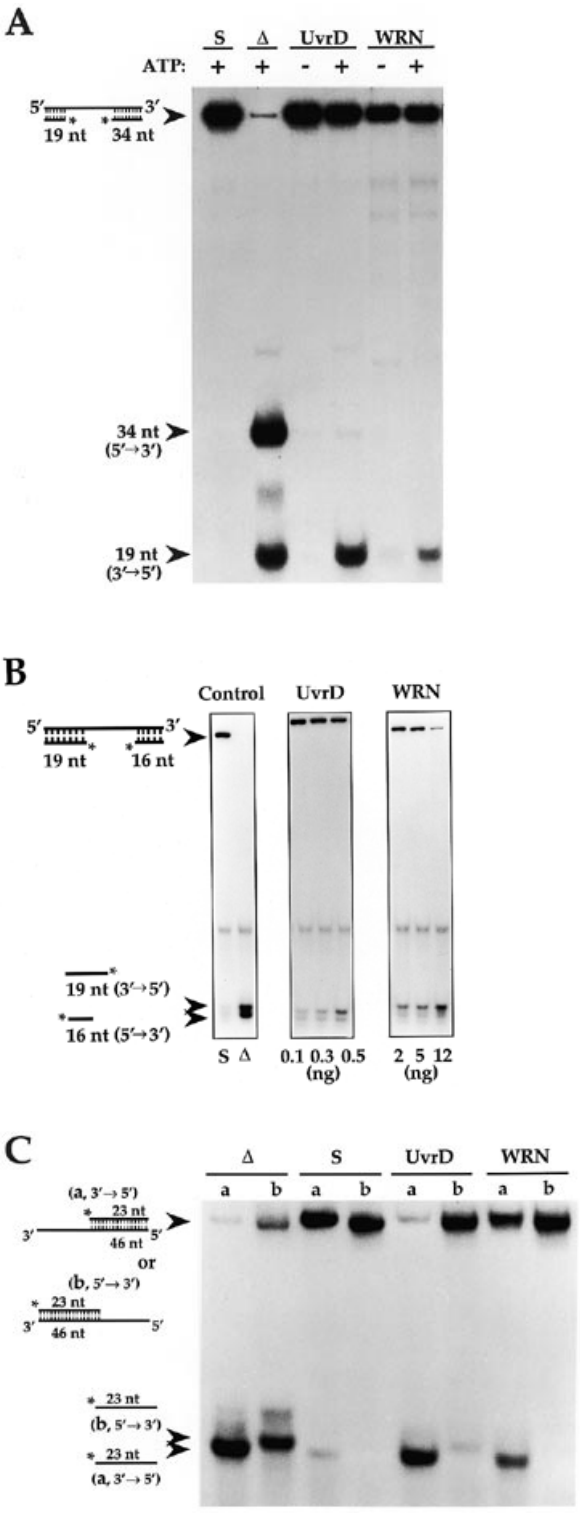
RESULTS

Directionality of WRN helicase

The direction of translocation of a DNA helicase is defined as the polarity of movement along the bound strand, i.e. either 3′→5′ or 5′→3′. Members of the RecQ family have been shown to unwind DNA predominantly in the 3′→5′ direction. We first employed a linear DNA substrate that consists of ssM13mp2 DNA with blunt-ended terminal-duplexes of 19 bp at the 5′-end and 34 bp at the 3′-end (Materials and Methods; 30). Displacement of the 19mer from the 5′ terminus indicates 3′→5′ polarity, and release of the 34mer from the 3′ terminus indicates 5′→3′ polarity. The WRN helicase displaced the 19mer but not the 34mer, suggesting 3′→5′ directionality (Fig. 1A). The 3′→5′ DNA helicase, UvrD (*E.coli* helicase II), showed a similar preference for displacing the 19mer. Omission of ATP from the reactions abolished strand

displacement by WRN as well as UvrD, again confirming the dependence of these helicase activities on ATP hydrolysis.

Since the WRN helicase has reduced activity in the displacement of a longer oligonucleotide, e.g. 53 bp (12), the reduction in unwinding in the 5′→3′ direction could be due to the greater length of the oligonucleotide hybridized to the 3′-terminus. We therefore constructed a similar linear ssM13mp2 DNA with a shorter oligonucleotide hybridized to the 3′-terminus (Materials and Methods). This substrate contains a 16 bp duplex at the 3′-end of the DNA, instead of a 34mer as in the previous case, and the same 19 bp duplex at the 5′-end. Incubation of this substrate with increasing concentrations of WRN, as well as with the UvrD control, resulted in preferential displacement of the 19mer (Fig. 1B), confirming that WRN helicase has 3′→5′ directionality. In order to reduce the possibility of sequence context being the determinant of directionality in these experiments, we constructed two related partial duplex oligonucleotides. The duplexes shared the same 46mer, but one had a complementary 23mer hybridized to the 3′ half and the other a complementary 23mer hybridized to the 5′ half. Thus the two substrates have similar structures and characteristics: blunt-ends, identical lengths of single-stranded tails (23 nt) and the same melting temperatures (66°C). Results of strand displacement with this pair of substrates by WRN and UvrD are shown in Figure 1C. Both WRN and UvrD displaced the labeled 23mer in the 3′→5′ direction, with UvrD alone displaying slight activity consistent with displacement from a blunt end.



NTP and dNTP dependence of WRN helicase activity

Known DNA helicases, including WRN (12) exhibit DNA-dependent ATPase activity, and the hydrolysis of nucleoside triphosphate supplies the energy for the DNA unwinding process. We therefore measured strand displacement activity of WRN with different NTPs and dNTPs. Both ATP and dATP supported the displacement of labeled 20mers in a 10 min incubation at 37°C; as quantified by PhosphorImager 36% of the duplex was separated in the presence of 1 mM ATP and 58% in the presence of 1 mM dATP (Fig. 2A). Also, strand displacement by 1 mM dCTP was 35%. In two separate experiments, the extent of strand dispalcement by 1 mM CTP was 16 and 42%. On the other hand, neither GTP, dGTP, UTP nor dTTP (1 mM) effectively substituted for ATP, strand displacement being <10% (Fig. 2A and data not shown). Our preliminary results suggest that the doublet in the displaced band in the dA, dC, A and C lanes in Figure 2A

Figure 1. Directionality of WRN helicase. (**A**) Autoradiogram of a 12% polyacrylamide gel showing reaction products derived from a partial duplex M13 DNA substrate. Reactions were carried out at room temperature for 1 h after adding UvrD (16 ng) or WRN (15 ng) to buffer containing 0.1 pmol of linearized M13mp2 DNA with blunt-ended partial duplex termini, in the presence or absence of 1 mM ATP. Displacement of a ^{32}P-labeled 34mer annealed to the 3′-end of the DNA indicates translocation in the 5′→3′ direction, while displacement of a ^{32}P-labeled 19mer annealed to the 5′-end indicates translocation in the 3′→5′ direction. S, substrate in the absence of helicase; Δ, heat-denatured substrate. (**B**) Autoradiogram of a 12% polyacrylamide gel showing products derived from a related M13 DNA substrate. Reactions were executed at 37°C for 10 min by incubating various concentrations of UvrD or WRN, 1 mM ATP and another linearized M13mp2 DNA substrate with blunt-ended partial duplex termini. A ^{32}P-labeled 16mer annealed to the 3′-end is displaced by 5′→3′ helicase activity, and a ^{32}P-labeled 19mer annealed to the 5′-end is displaced by 3′→5′ helicase action. (**C**) Autoradiogram of a 12% polyacrylamide gel showing reaction products derived from a pair of oligomer substrates. Each blunt-ended partial duplex oligonucleotide substrate (0.1 pmol) was incubated with 16 ng of UvrD or 15 ng of WRN in reaction buffer containing 1 mM ATP at 37°C for 10 min. The 3′→5′ helicase substrate has a complementary ^{32}P-labeled 23mer annealed to the 5′-end of a 46mer. The 5′→3′ helicase substrate has a different complementary ^{32}P-labeled 23mer annealed to the 3′-end of the 46mer.

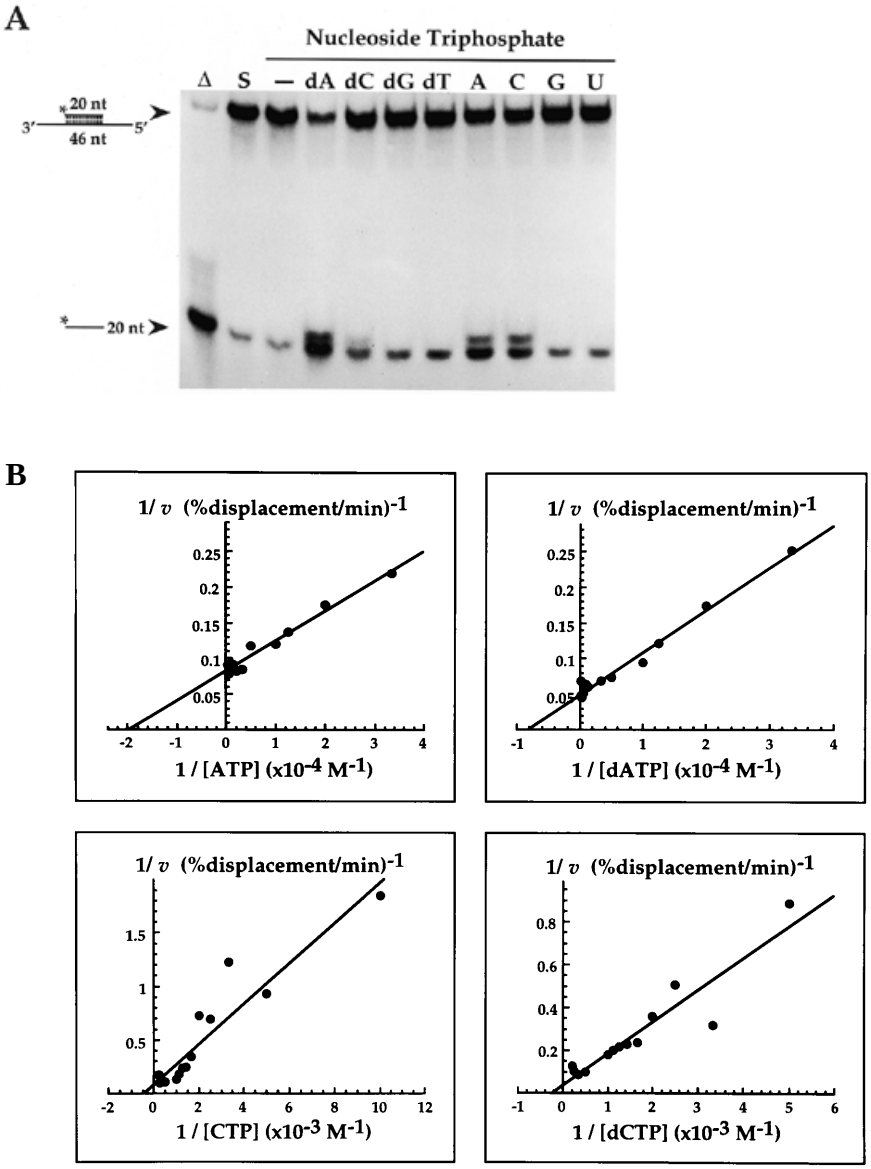


Figure 2. Dependence of WRN helicase activity on nucleoside triphosphates. (**A**) Autoradiogram of a 12% polyacrylamide gel with electrophoretically separated reaction products derived from incubating WRN with an oligonucleotide substrate in the presence of different NTPs or dNTPs. The substrate (0.1 pmol), a ^{32}P-labeled 20mer hybridized to a 46mer, was incubated with 15 ng of WRN in reaction buffer containing 1 mM NTP or dNTP at 37°C for 10 min. Δ, heat-denatured substrate; S, substrate in the absence of a helicase. (**B**) Lineweaver–Burk plots for ATP, dATP, CTP and dCTP. Strand displacement reactions were carried out with various concentrations of NTP as in (A) except that reactions with ATP and dATP were incubated for 3 min and reactions with CTP and dCTP were incubated for 6 min. Reaction products analyzed by electrophoresis on a 12% polyacrylamide gel were vacuum dried and quantitated by PhosphorImager analysis.

represents the presence of an exonucleolytic activity inherent in WRN. The presence of a 3′→5′ exonuclease encoded within WRN has been predicted by Mushegian *et al.* (31) and Moser *et al.* (32).

Kinetic constants for reactions with ATP, dATP, CTP and dCTP were determined from Lineweaver–Burk plots (Fig. 2B). Reactions with ATP and dATP were incubated at 37°C for 3 min; the % displacement was a linear function of time during this interval (data not shown). For CTP and dCTP, the incubation was 6 min, and displacement was also linear with time (data not shown). The K_m and V_{max} values are shown in Table 1. The K_m for ATP (51 μM) was lower than for dATP (119 μM), but the V_{max} (20% displacement/min) for dATP was higher than for ATP (12% displacement/min). The measure of relative catalytic efficiency, V_{max}/K_m was 0.24% displacement μM^{-1} min^{-1} for ATP and 0.17% displacement μM^{-1} min^{-1} for dATP, indicating a near-equivalent efficiency for both nucleoside triphosphates as substrates for WRN. K_ms for CTP (2.1 mM) and dCTP (3.9 mM) were much higher than for either ATP or dATP, suggesting that ATP and dATP are two major energy sources for WRN helicase activity *in vivo*.

Effects of SSBs on the strand displacement of WRN helicase

Single-stranded DNA-binding proteins have been shown to enhance DNA unwinding by different DNA helicases *in vitro* (33–35). We previously reported that addition of *E.coli* SSB facilitates the displacement of a long oligonucleotide (e.g. 53mer)

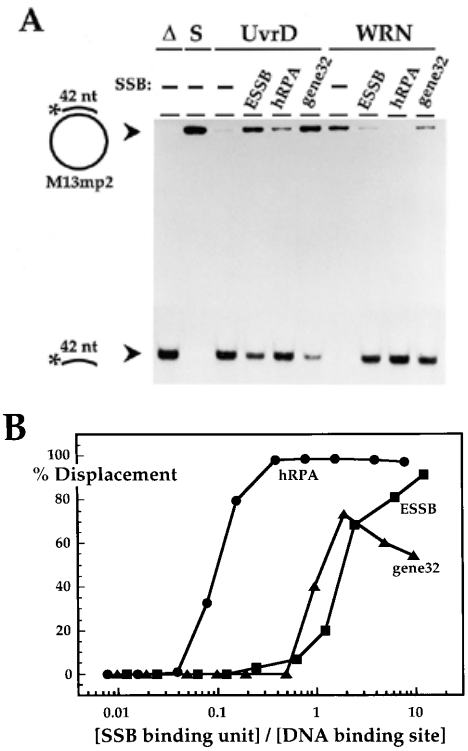


Figure 3. SSB effects on the strand displacement activity of WRN. (**A**) Autoradiogram of a 12% polyacrylamide gel showing the reaction products derived from incubating WRN and a DNA substrate in the absence or presence of various SSBs. The substrate (1 fmol), a ^{32}P-labeled 42mer hybridized to the circular M13mp2 DNA, was incubated with 16 ng of UvrD or 15 ng of WRN for 1 h at room temperature in reaction buffer containing either no SSB or 0.3 μg of *E.coli* SSB (ESSB), 0.3 μg of human RPA (hRPA) or 0.1 μg of T4 gene 32 protein (gene 32). Δ, heat-denatured substrate; S, substrate. (**B**) Concentration-dependence of SSB effects on the strand displacement activity of WRN. Reactions were carried out as in (A), with various concentrations of each SSB. Reaction products were resolved in 12% polyacrylamide gels and were subjected to quantitation by PhosphorImager. The data were plotted as % of total DNA substrate displaced as a function of SSB binding unit per DNA binding site (see text for details).

from a partial duplex ssM13mp2 DNA by WRN (12). In order to compare the stimulation of WRN helicase activity by different SSBs, we utilized a 42mer annealed to ssM13mp2 DNA as a substrate. In the absence of SSB proteins, WRN was unable to significantly displace this oligonucleotide (Fig. 3A). However, upon the addition of *E.coli* SSB (ESSB), human replication protein A (hRPA) or bacteriophage T4 gene 32 protein (gene 32) the oligonucleotide was displaced from the hybrid (Fig. 3A). Using similar concentrations of hRPA (0.28 μM of heterotrimer), *E.coli* SSB (0.38 μM of tetramer) or T4 gene 32 (0.3 μM of monomer), the extent of strand displacement was greatest with hRPA. In contrast, strand displacements by UvrD was inhibited by each of the SSB proteins under the same conditions in which stimulation was observed with WRN protein.

To further understand the relationship between WRN and each SSB during DNA unwinding, strand displacement was measured as a function of the concentration of hRPA, *E.coli* SSB and T4 gene 32 protein (Fig. 3B). Since the functional units of different SSBs—monomer versus multimer—cover different numbers of nucleotides when bound to ssDNA, we indicated the concentration of each SSB as the ratio of binding units (molar concentration of functional unit) per DNA binding site (concentration of ssDNA in nucleotides divided by the number of nucleotides covered per unit). For example, 1 on the *x*-axis in Figure 3B indicates an amount of SSB sufficient, if all is bound, to cover the entire single-stranded template. Under the reaction conditions, the binding site size for the hRPA heterotrimer is ~30 nt (36,37), for the *E.coli* SSB tetramer ~35 nt (38,39) and for the T4 gene 32 monomer ~7 nt (38,40). Figure 3B clearly illustrates that hRPA stimulated strand displacement most efficiently. With hRPA, maximum stimulation was observed at a concentration three times less than that required to cover all template sites, whereas with *E.coli* SSB and T4 gene 32 displacement was only 15 and 40% at concentrations required to saturate all single-stranded binding sites. To attain 50% displacement, the concentration (in functional units) of hRPA required was ~10 or 20 times less than that of T4 gene 32 or *E.coli* SSB, respectively. Moreover, the curves for the three SSBs are quite different; the hRPA curve appears hyperbolic, while the *E.coli* SSB curve has a sigmoidal form and the T4 gene 32 curve shows inhibition at concentrations 2-fold greater than that required for saturation of binding sites. These observations suggest a specific, biologically relevant interaction between hRPA and WRN that results in the stimulation of WRN helicase activity. This stimulation is different from that caused by *E.coli* SSB and T4 gene 32 which may involve solely interactions with the single-stranded template.

DISCUSSION

In order to gain insights into the role of WRN helicase in maintaining genetic stability, we analyzed the directionality of unwinding, NTP utilization and effects of ssDNA-binding proteins. First we determined that the direction of translocation of WRN is 3′→5′ on the ssDNA portion of partially duplex substrates. This confirms our preliminary observations (12) and is characteristic of DNA helicases that belong to the RecQ family. *E.coli* RecQ, the first studied member of this family, was initially identified as a 3′→5′ DNA helicase involved in homologous recombination via the RecF pathway (14). Other studies have suggested a role for RecQ in the suppression of illegitimate recombination in *Escherichia coli* (15) as well as in the re-initiation of damage-impeded DNA synthesis at the replication fork (16,17). If a RecF-like damage-response pathway is present in mammalian cells and functions in the resolution of DNA damage at the replication fork, this could account for the sensitivity of WS cells to a limited number of DNA damaging agents. Peripheral blood lymphocytes from WS patients (41) and SV40-transformed WS cells (42) are hypersensitive to 4-nitroquinoline-1-oxide (4-NQO), but not to a variety of other DNA damaging agents. The finding of increased chromosome breakage induced by 4-NQO (41) initially suggested that WRN protein may play a role in a specific recombinational DNA repair pathway in mammalian cells, but is equally compatible with a role for WRN in the repair or bypass of DNA damage ahead of the replication fork or in the repair of double-strand breaks. The intermediate sensitivity to 4-NQO of cells from WS heterozygotes (42) suggests that deficits in WRN may have functional significance beyond WS itself, and may be relevant to genetic instability and the incidence of specific human tumors in the population at large.

Recently, BLM (the gene product mutated in BS), another RecQ homolog, has also been expressed and determined to unwind DNA in the 3′→5′ direction (19). Mutations in both genes are

associated with genetic instability and with a proclivity to the development of cancer. However, the types of associated cancers, other clinical symptoms and the behavior of cells in culture are entirely different (43). The enhancement in sister chromatid exchange that is characteristic of BS is not observed in WS. These differences in phenotype may indicate that the two RecQ homologs WRN and BLM function in different pathways of DNA metabolism in cells.

Table 1. Kinetic values for the NTP and dNTP cofactors of WRN

	ATP	dATP	CTP	dCTP
K_m	51 μM	119 μM	21 mM	3.9 mM
V_{max} (%/min)[a]	12	20	11	26

K_m and V_{max} values for ATP, dATP, CTP and dCTP were determined from the Lineweaver–Burk plots in Figure 2B.
[a]% of the total DNA substrate displaced/min.

We also measured the ability of each of the eight common NTPs and dNTPs to serve as cofactors for WRN DNA helicase activity. ATP, dATP, CTP and dCTP can support strand-displacement. The V_{max} values for strand-displacement with these nucleoside triphosphates are very similar. However, the lower K_m values for ATP and dATP suggest that these are more likely to serve as an energy source *in vivo*. The preferential utilization of ATP or dATP could be a general characteristic of the RecQ family, since *E.coli* RecQ was demonstrated to use both ATP and dATP efficiently, but not GTP (44). In addition, human helicase α, composed of 110 and 90 kDa polypeptides, utilizes ATP and dATP for DNA unwinding. The K_m values obtained with human helicase α for ATP and dATP are 28 and 48 μM, respectively, not greatly dissimilar from the values of 51 and 119 μM for ATP and dATP, respectively, obtained with WRN. In fact, there are many mammalian DNA helicases that use ATP and dATP as major energy sources and translocate in a 3′→5′ direction (34,45–48). The ability of WRN to effectively use CTP and dCTP as substrates suggests that these nucleotides might assist in identifying WRN helicase activity in crude cell extracts, even though these substrates might not be physiologically relevant.

Lastly, we studied the ability of different ssDNA-binding proteins to stimulate WRN helicase activity. Mechanistically, SSBs could bind to ssDNAs during strand displacement reactions, and prevent the displaced ssDNAs from re-hybridizing with the DNA template. On the other hand, high concentrations of SSB might inhibit strand displacement by competing with helicase for binding at the junction of single- and double-stranded DNA, if there is no specific coordination between the two proteins. We observed stimulation of WRN-catalyzed strand displacement by the three SSBs we tested, although to different degrees (Fig. 3B). The helicase activity of *E.coli* RecQ is also stimulated by *E.coli* SSB and T4 gene 32 protein (33). In contrast, we observed inhibition of UvrD-catalyzed strand displacement by all three SSBs (Fig. 3A), reflecting the fact that SSB is not required for UvrD to unwind DNA and actually impedes the unwinding process.

Of the three SSBs we tested, human RPA was the most effective in enhancing WRN helicase activity. For example, 10–20 times higher effective concentrations of *E.coli* SSB or T4 gene 32 protein than of hRPA were required to achieve the same extent of stimulation. Moreover, the concentration dependence of stimulation (Fig. 3B) followed a hyperbolic curve in the case of hRPA and a sigmoidal curve in the case of *E.coli* SSB. The sigmoidal curve observed for *E.coli* SSB probably reflects cooperative binding to ssDNA that prevents reannealing of the displaced oligomer. The hyperbolic curve found for hRPA in two separate experiments may reflect a non-cooperative, direct interaction between WRN and hRPA, since the cooperativity (ω) of hRPA is much less than that of *E.coli* SSB (ω_{RPA} = 10–20 versus $\omega_{E.coli\ SSB} = 1 \times 10^5$) (38,49), and cooperative binding could result in a sigmoidal stimulation curve. Additional studies will be required to determine whether hRPA interacts directly with WRN protein. There is other evidence which may indicate that SSB proteins interact with DNA helicases. The ability of *E.coli* RecQ to separate long hybridized oligonucleotides is increased by the addition of *E.coli* SSB, e.g. the combination can efficiently unwind a 71 bp partial duplex (33). The strand-displacement activity of human helicase α is increased in the presence of hRPA (35). Interestingly, DNA helicases have been purified from mouse cell extracts by RPA affinity chromatography (50). Thus, the stimulation of WRN helicase by hRPA may indicate a direct interaction between these proteins.

Human SSB (hRPA) was initially shown to be a DNA replication protein (37) and to serve a function in nucleotide excision repair as well (51). Studies in yeast also indicate that RPA facilitates nucleation of ssDNA by Rad51 and is thus involved in homologous recombination (52). Recently, human RPA was shown to facilitate homologous pairing and strand transfer reactions induced by human Rad51 (53). Moreover, RPA interacts with the tumor suppressor protein p53 (54) and is functionally regulated by ATM-dependent phosphorylation (55–57). All of these findings suggest multiple roles for RPA and therefore lead to the speculation that WRN may be involved in one or more of these RPA-associated DNA metabolic processes.

Genetic instability in WS was initially demonstrated by the presence of multiple clones with different translocations among cells from the same individual (variegated translocational mosacism) (8). This instability was confirmed by studies of WS fibroblasts in culture that demonstrated a 50-fold enhancement in the rate of *hprt* mutagenesis. Examination of the DNA sequence of the mutants indicated that the most frequent types of mutations were extensive deletions (9). Studies in yeast have shown that mutations in RPA result in an elevated mutation rate that is also characterized by deletions (R.Kolodner, personal communication). Thus, the interaction we observed between hRPA and WRN may reflect the finding that mutations observed in WS patients are predominantly deletions.

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