The human premature aging disorder Werner syndrome (WS) is associated with a large number of symptoms displayed in normal aging. The WRN gene product, a DNA helicase, has been previously shown to unwind short DNA duplexes (≤53 base pairs) in a reaction stimulated by single-stranded DNA-binding proteins. We have studied the helicase activity of purified WRN protein on a variety of DNA duplex substrates to characterize the unwinding properties of the enzyme in greater detail. WRN helicase can catalyze unwinding of long duplex DNA substrates up to 849 base pairs in a reaction dependent on human replication protein A (hRPA). *Escherichia coli* SSB and bacteriophage T4 gene 32 protein (gp32) completely failed to stimulate WRN helicase to unwind long DNA duplexes indicating a specific functional interaction between WRN and hRPA. So far, there have been no reports of any physical interactions between WRN helicase and other proteins. In support of the functional interaction, we demonstrate a direct interaction between WRN and hRPA by coimmunoprecipitation of purified proteins. The physical and functional interaction between WRN and hRPA suggests that the two proteins may function together in vivo in a pathway of DNA metabolism such as replication, recombination, or repair.

WS¹ is a human autosomal recessive disease described as a “segmental progeroid syndrome” because it manifests signs of normal aging (1). Prevalent disorders of WS, including osteoporosis, atherosclerosis, diabetes mellitus (type II), cataracts, wrinkled skin, and gray hair, develop in early adulthood. In addition, cancers, particularly sarcomas, have been observed in these patients with increased frequency. Most patients afflicted with WS die before age 50.

Somatic cells of WS patients display a mutator phenotype characterized by extensive chromosomal rearrangements (2) and deletions (3). WS cells exhibit a reduced replicative life span (4, 5) and show some defects in DNA synthesis. Specifically, some WS cells have an extended S phase (6) and a reduced frequency of initiation sites (7, 8). In addition to replication, some WS cells display a hyper-recombinogenic phenotype (9) which may contribute to genomic instability. A possible defect in recombination is supported by the demonstration that WRN homologs in yeast sgs1 (*Saccharomyces cerevisiae*) (10, 11) and rqh1 (*Schizosaccharomyces pombe*) (12), bacteria recQ (13), and human BLM (14) all negatively regulate recombination. Notably, the human WRN gene can suppress the increased homologous and illegitimate recombination in sgs1 (15).

Some evidence suggests a role for the WRN protein in DNA repair. This notion is supported by a recent report that WS cells are hypersensitive to the carcinogen 4-nitroquinoline 1-oxide (6, 17) which introduces bulky base damage, as well as 8-hydroxyguanine and strand breaks (18). However, WS cells do not exhibit a hypersensitivity to other DNA-damaging agents suggesting that a repair deficiency in WS is subtle and perhaps not the primary defect in the disease. This laboratory using the gene-specific and strand-specific repair assays reported a fine structure defect in WS lymphoblasts (19). Some WS cells also exhibit reduced telomere repair (20). In addition to DNA metabolic defects, evidence from this laboratory suggests that WS cells are partially defective in transcription.² Further studies will be required to test hypotheses directly implicating WRN protein in specific DNA metabolic pathways.

The gene defective in WS, designated *WRN*, encodes a protein of 1432 amino acids with the seven conserved motifs found in a class of enzymes known as helicases (21). By sequence alignment, the *WRN* gene product belongs to the RecQ superfamily of DNA helicases that includes *Escherichia coli* RecQ, *S. cerevisiae* Sgs1p, *S. pombe* rqh1p, and two human proteins, BLM (defective in Bloom syndrome) and RecQL. The WRN protein has been shown to be a DNA-stimulated ATPase and a helicase (22, 23), catalyzing strand displacement of short oligonucleotides (≤53 bp) with a 3′ to 5′ polarity (22, 24). In addition to the helicase domains, the protein sequence of WRN contains a region of similarity to the 3′ to 5′ exonuclease domain of *E. coli* DNA polymerase I and RNase D (25) and has been shown to possess a 3′ to 5′ exonuclease activity (26–28). Aside from these basic biochemical activities, molecular details of the interactions of WRN protein with other proteins and biological DNA substrates remain to be well defined. The molecular deficiencies involved in the clinical phenotype of WS presumably reflect an impaired function of the WRN protein in a pathway of nucleic acid metabolism.

In this study, we have investigated a possible interaction between WRN protein and hRPA. hRPA, composed of three subunits 70, 32, and 14 kDa, was originally isolated as a factor required for simian virus (SV40) DNA replication *in vitro* (29).

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hRPA binds tightly to ssDNA and only weakly to dsDNA or RNA. hRPA interacts specifically with SV40 large T antigen (a 3’ to 5’ DNA helicase) and DNA polymerase α (30). In addition to its role in viral replication, hRPA is required for replication of chromosomal DNA. hRPA has also been directly implicated in the following three steps of nucleotide excision repair: damage recognition (31), excision (32), and gap-filling DNA synthesis (33–35). Recently it was shown that hRPA plays a role in long patch DNA base excision repair (36). RPA is proposed to play a role in recombination by stimulating strand exchange proteins from S. cerevisiae (37) and humans (38). These findings indicate that hRPA is a key player in DNA metabolism, modulating these processes by protein-protein and protein-DNA interactions.

As shown previously by Shen et al. (24), the helicase activity of WRN protein on a 42-bp partial duplex is stimulated by several SSBs. In their experiments, the helicase activity of WRN protein was stimulated better by hRPA than either ESSB or gp32. We have further examined the WRN-hRPA connection by characterizing the effect of SSBs on WRN helicase activity using DNA substrates of various lengths. ESSB and gp32 completely failed to stimulate WRN helicase activity on long DNA duplexes. In contrast, hRPA was required to support WRN helicase activity on long DNA duplexes indicating a functional interaction between WRN and hRPA. This functional interaction was further substantiated by the demonstration of a physical interaction between hRPA and WRN. This interaction, the first to be described for WRN protein, is likely to play a critical role in a pathway of DNA metabolism defective in WS.

**Materials and Methods**

**WRN Protein Purification**—A recombinant hexa-histidine-tagged WRN protein was overexpressed in Sf9 insect cells and purified by Ni²⁺ chromatography as described previously (22) with some modifications. All steps of purification were carried out at 4 °C. Briefly, insect cells were resuspended in Lysis Buffer (150 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor mixture (2 μg/ml pepstatin, leupeptin, aprotinin, and chymostatin)) and incubated for 15 min. Insoluble material was removed by centrifugation at 10,000 × g. The clarified lysate was bound in columns to nickel-agarose resin (Qiagen), pre-equilibrated in Lysis Buffer. The resin/lysate slurry was transferred to a column, and unbound material was allowed to flow through. The column was sequentially washed with 20 column volumes of Wash Buffer 1 (150 mM Tris (pH 8.0), 0.5 mM LiCl, 10% glycerol, 5 mM β-mercaptoethanol, 1 mM PMSF, and protease inhibitor mixture), 10 column volumes of Wash Buffer 2 (10 mM PIPES (pH 7.0), 50 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol, 1 mM PMSF, and protease inhibitor mixture), 10 column volumes of Wash Buffer 3 (Wash Buffer 2 plus 10 mM imidazole), and 20 column volumes of Wash Buffer 4 (Wash Buffer 2 plus 25 mM imidazole) to remove nonspecifically bound protein. WRN protein was eluted from nickel-agarose resin using Elution Buffer (10 mM PIPES (pH 7.0), 50 mM NaCl, 300 mM imidazole, 20% glycerol, 5 mM β-mercaptoethanol, and 1 mM PMSF). The solubility of WRN protein was maintained by immediate addition of BSA to a final concentration of 100 μg/ml. Purified recombinant WRN protein (1.5 μg) migrated as a single Coomassie-stained band on an SDS-polyacrylamide gel (Fig. 1A). Protein from cells infected with baculovirus containing WRN-K577M band on an SDS-polyacrylamide gel (Fig. 1B). Beads were stored at 4 °C for 2 h. For immunoprecipitation experiments, 1 μg of purified hRPA was incubated with or without 1 μM purified WRN protein and incubated at 24 °C. Samples (5 μl) were removed at 1- or 2-min intervals and evaluated by thin layer chromatography as described previously (42). Less than 20% of the substrate ATP was consumed in the reaction over the entire course of the experiment.

**Immunoprecipitation**—Affinity beads coated with WRN antibody were used to immunoprecipitate WRN-hRPA complexes. ScFvs against WRN have been selected from a phage display library. Two different WRN monoclonal scFvs were coated on tosyl-activated magnetic beads (Dynal) according to the manufacturer’s protocol. Briefly, the tosyl-activated beads were washed twice in 100 mM sodium phosphate buffer (pH 7.4) and incubated with 500 μl of purified scFv (200 μg of scFv, 10% glycerol, 100 mM NaCl, 50 mM sodium phosphate (pH 7.2)) for 24 h at 24 °C. Beads were washed twice in buffer B (150 mM NaCl, 100 mM sodium phosphate (pH 7.4), 0.1% BSA) and incubated in buffer E (100 mM Tris (pH 8.5), 0.5% BSA) for 24 h at 24 °C. Beads were stored at 4 °C after washing in buffer D. For immunoprecipitation experiments, 1 μg of purified hRPA was incubated with or without 1 μM purified WRN protein in 200 μl of helicase reaction buffer (40 mM Tris (pH 7.4), 5 mM dithiothreitol, 4 mM MgCl₂) containing 2 mM ATP and 5 μg/ml BSA at 4 °C for 90 min. Approximately 3.3 × 10⁶ WRN antibody-coated affinity beads in 20 μl of helicase buffer were added, and the mixture was incubated for 1 h at 4 °C. The beads were isolated 5 times in 400 μl of helicase buffer containing 150 mM NaCl and 0.1% BSA. Immunoprecipitated material was resuspended in 20 μl of 2× SDS loading buffer and incubated at 95 °C for 2 min. The samples were run on 8–16% SDS-polyacrylamide gel electrophoresis and electropho...
onto nitrocellulose membranes using the NOVEX system. hRPA was detected using a monoclonal mouse IgG against hRPA (Calbiochem) and goat anti-mouse IgG-horseradish peroxidase (Sigma). WRN was detected using a goat polyclonal IgG against WRN (Santa Cruz Biotechnology) and donkey anti-goat IgG-horseradish peroxidase (Santa Cruz Biotechnology). Both WRN and hRPA were detected by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech).

RESULTS

Biochemical Properties of Wild-type and Mutant WRN Protein—The recombinant WRN protein was purified using nickel-agarose as described under “Materials and Methods.” The inclusion of an extensive wash with buffer containing low imidazole concentrations (10–25 mM) effectively removes a number of residual contaminant proteins from the final WRN protein preparation. Recombinant WRN protein migrated as a single Coomassie-staining band of the predicted size (~163 kDa) on a 8–16% polyacrylamide gel run in the presence of SDS (Fig. 1A). Purified WRN protein was judged to be pure of DNA by analysis using SYBR Green Stain (FMC Bioproducts).

To ascertain the enzymatic purity of the WRN protein, ATPase and helicase assays were conducted on the wild-type and mutant WRN-K577M proteins purified in an identical manner. In the WRN-K577M mutant protein, the invariant lysine residue of the conserved motif I (Walker A ATPase site) of DNA helicases has been replaced with a methionine (22). On a short 28-bp partial duplex DNA substrate, wild-type WRN protein unwound approximately 80% of the substrate molecules in 60 min (Fig. 1B, lane 1), whereas no unwinding of the 28-bp partial duplex by WRN-K577M was detected (Fig. 1B, lane 2). We also examined the ATPase activity of the purified wild-type and mutant WRN proteins (Fig. 1C). The $k_{cat}$ of wild-type WRN protein for ATP hydrolysis using M13mp18 as the ssDNA effector was 58.4 ± 17 min$^{-1}$. Importantly, the WRN-K577M mutant protein, purified using the same method as the wild-type WRN protein, exhibited no ATP hydrolysis.

Thus WRN protein is devoid of contaminating helicase and ATPase activities and suitable for use in these studies.

The $k_{cat}$ value of 58.4 min$^{-1}$ for WRN-catalyzed ATP hydrolysis using M13mp18 ssDNA as the DNA effector obtained in these studies is dramatically different from the previously reported $k_{cat}$ value for WRN-catalyzed ATP hydrolysis of approximately 1,000,000 min$^{-1}$ using calf thymus DNA as the effector in the ATPase reaction (23). We have determined $k_{cat}$ values for WRN ATPase activity using a variety of ssDNA effectors that are all in the range of 50–200 min$^{-1}$ (Table I). We cannot explain the discrepancy between our data and the previously published data. To our knowledge, a $k_{cat}$ value of 1,000,000 min$^{-1}$ is far greater than any previously reported value for a DNA helicase, whereas a $k_{cat}$ of 58 min$^{-1}$ is in the same range as values reported for a number of DNA helicases (for review, see Matson et al. (43)).

Characterization of Helicase Activity—An important property of DNA helicases is their ability to unwind DNA duplexes of various lengths. A highly processive helicase is required in the process of replication where long stretches of base pairs must be unwound in an efficient manner. In contrast, a helicase capable of unwinding short DNA duplexes could serve in short patch DNA repair. By using a variety of DNA substrates, biochemical studies of purified helicases in vitro have demonstrated that each DNA unwinding enzyme exhibits its own characteristic dependence of unwinding on DNA duplex length (43). To characterize the effect of duplex length on the unwinding activity of the WRN helicase, we tested partial duplex substrates of varying lengths in a strand displacement assay. WRN helicase alone readily catalyzed 80% unwinding in 60 min on a short partial duplex of 28 bp (Fig. 1B). Thus WRN helicase catalyzes unwinding of very short DNA duplexes confirming previous reports (22–24).

We subsequently analyzed WRN helicase activity on longer DNA duplex substrates in the presence or absence of SSB. WRN-catalyzed unwinding of a 69-bp partial duplex required an SSB (Fig. 2A). WRN protein (184 nM) alone failed to catalyze detectable unwinding of the 69-bp partial duplex DNA substrate. In control reactions, hRPA (384 nM, heterotrimer) or ESSB (1456 nM, homotetramer) alone did not denature the 69-bp partial duplex DNA substrate. Either ESSB or hRPA could stimulate WRN helicase to unwind the 69-bp partial duplex. However, WRN helicase activity on the 69-bp partial duplex is stimulated much more efficiently by hRPA than ESSB. In the hRPA-supported reaction, approximately 95% of the 69-bp partial duplex is unwound at a concentration of 96 nM hRPA heterotrimer (Fig. 2B). In the WRN helicase reaction containing ESSB, a maximum of 20% unwinding of the 69-bp partial duplex is achieved at a concentration of 1092 nM ESSB homotetramer. Thus a 10-fold higher molar concentration of ESSB compared with hRPA only modestly supports WRN helicase unwinding of the 69-bp duplex, approximately one-fifth the level observed in the hRPA-supported reaction.

SSB may stimulate helicase activity by simply coating the ssDNA generated during the unwinding reaction, thereby preventing reannealing of the two displaced strands. To gain insight into the mechanism of stimulation of helicase activity by SSB, strand displacement was expressed as a function of the ratio (R) of SSB-binding units per DNA-binding site (Fig. 2C). This analysis takes into account the fact that one ESSB homotetramer binds 35 nt (44) and one hRPA heterotrimer binds 30 nt (29). A comparison of the ESSB and hRPA profiles using the 69-bp partial duplex substrate shows that at a concentration of hRPA which coats the ssDNA molecules in the helicase reaction (96 nM heterotrimer), 95% of the 69-mers are released by WRN helicase. In contrast, at an ssDNA coating concentration of ESSB (91 nM homotetramer), only 6% of the 69-mers are released. These results are in good agreement with a published report showing a similar difference between the hRPA and ESSB-supported WRN helicase reactions on a shorter partial duplex of 42 bp at an R value of 1 (24). However, results with the 42-bp partial duplex show that at an RESS$^{−1}$ value of 10, approximately 90% of the 42-mers were released, whereas we observe only 20% displacement of the 69-bp partial duplex at an RESS$^{−1}$ value of 25. This difference most likely reflects the increased duplex length of 27 bp (69 versus 42 bp) suggesting that ESSB, even at high R values, functions poorly with WRN helicase on the slightly longer duplex.

A kinetic analysis of the WRN-catalyzed unwinding reaction on the 69-bp partial duplex demonstrated a dramatic difference in the effect of hRPA versus ESSB on the rate of helicase activity (Fig. 3A). In this experiment, we used concentrations of hRPA (96 nM, heterotrimer) or ESSB (91 nM, homotetramer) sufficient to coat the ssDNA-binding sites of the partial duplex DNA substrate (R = 1). Nearly all of the 69-bp partial duplex (97%) was unwound by WRN (184 nM) in 40 min in the hRPA-supported reaction (Fig. 3B). At later time points of the incubation, a reduced amount of released fragment was observed most likely reflecting nucleosome activity intrinsic to WRN. By using hRPA, the initial rate of helicase activity during the first 20 min was determined to be 4.6% of the 69-bp partial duplex substrate unwound per min. As shown previously in Fig. 2, ESSB performed poorly in the stimulation of WRN helicase unwinding. Only a small fraction of the 69-bp helicase substrate (~3%) was unwound in the reaction containing ESSB in 120 min. The inability to detect unwinding in the ESSB-supported reaction during the first 40 min prevented us from
comparing initial rates of the reactions between hRPA and ESSB. However, the initial rate of the hRPA-supported reaction was 186-fold greater than the overall rate of the ESSB-supported reaction. Thus, at ssDNA coating concentrations of SSB, the WRN helicase functions far more efficiently with hRPA than with ESSB on the relatively short 69-bp partial duplex DNA substrate.

To address further the issue of specificity in the functional interaction between WRN and hRPA, we tested the ability of gp32, an SSB from bacteriophage T4, to stimulate WRN helicase activity on the 69-bp partial duplex DNA substrate. Only a small amount of unwinding (~2%) could be detected in the presence of gp32 concentrations up to 6042 nM (monomer), a 21-fold excess of gp32 binding equivalents over gp32-binding sites based on the ssDNA-binding site size of gp32 (7 nt/monomer) (44) (Fig. 4). Thus gp32, like ESSB, performs poorly in the WRN helicase reaction indicating that the hRPA-WRN interaction is specific.

Previously, the longest DNA duplex reported to be unwound by WRN helicase was 53 bp (22). To examine further the effect of duplex length on WRN helicase activity, a 257-bp partial duplex DNA substrate was tested for unwinding in the presence of hRPA or ESSB (Fig. 5A). As expected, there was no detectable unwinding of this longer duplex substrate by WRN alone. WRN-catalyzed displacement of the 257-mer in the hRPA-supported reaction could be detected at hRPA concentrations as low as 96 nM (heterotrimer) (Fig. 5B). A 1.5-fold increase in hRPA concentration (144 nM, heterotrimer) resulted in stimulating WRN helicase activity to 37% substrate unwound. Maximal unwinding of the 257-bp partial duplex (57%) was achieved at an hRPA concentration of 288 nM heterotrimer. In contrast, no unwinding of the 257-bp partial duplex was detected at ESSB concentrations up to 1456 nM (homotetramer), a 25-fold excess of ESSB binding equivalents over ESSB-binding sites (Fig. 5C). Likewise, no WRN-catalyzed unwinding of the 257-bp partial duplex could be detected in the presence of gp32 concentrations up to 6042 nM (monomer) (data not shown). The fact that both ESSB and gp32 completely failed to support unwinding of the long DNA duplex suggests that a specific interaction between WRN helicase and hRPA is responsible for unwinding the long 257-bp DNA duplex. The unique requirement for hRPA to stimulate WRN-catalyzed unwinding of a long DNA duplex indicates that this functional interaction is likely to be important in vivo.

To explore the possibility that WRN helicase can unwind substantially longer DNA duplexes, an 849-bp partial duplex DNA substrate was tested (Fig. 6A). WRN helicase alone or in the presence of ESSB or gp32 failed to catalyze unwinding of the 849-bp partial duplex DNA substrate (data not shown). A kinetic analysis of the WRN helicase reaction in the presence of hRPA demonstrated displacement of the 849-mer in a time-dependent manner (Fig. 6B). ATP hydrolysis catalyzed by the wild-type and K577M mutant WRN proteins. DNA-dependent ATPase assays using M13mp18 ssDNA were conducted as described under “Materials and Methods.” Reactions (30 μl) contained 0.80 mM [3H]ATP and 107 nM WRN (●) or 107 nM WRN-K577M (○). Reactions were initiated by the addition of protein, and aliquots (5 μl) were removed at 2-min intervals and processed as described previously (42).

![Image](https://example.com/image.png)
pendent manner (Fig. 6B). 14% of the 849-bp partial duplex substrate was unwound in 1 h, and approximately twice as much substrate (31%) was unwound in 2 h. Thus WRN helicase displaces duplexes ranging from 28 to 849 bp, but the efficiency of the reaction is dependent on the length of the duplex to be unwound, as well as the presence of an SSB. Importantly, WRN protein is capable of unwinding long tracts of DNA duplex in an hRPA-dependent reaction.

**Effect of hRPA on ATPase Activity**—To address the mechanism of hRPA stimulation of WRN helicase activity, we measured the effect of hRPA on ssDNA-dependent ATPase activity of WRN (Table I). The turnover rate constant \( k_{\text{cat}} \) for ATP hydrolysis by WRN helicase was determined at various concentrations of hRPA protein and compared with the \( k_{\text{cat}} \) value obtained in the absence of hRPA. As shown in Table I, there was no effect of hRPA on \( k_{\text{cat}} \) values for WRN-catalyzed ATPase activity using two DNA effectors, M13mp18 ssDNA circles or \((dT)_{263}\). We conclude that the stimulation of WRN helicase activity by hRPA is not due to increased efficiency in hydrolyzing ATP.

**Effect of Competitor DNA on Helicase Activity**—To investigate further the notion that hRPA may enhance the processivity of WRN helicase on DNA, we examined the effect of competitor DNA on the unwinding of a 28-bp partial duplex by WRN in the presence or absence of hRPA (Fig. 7). Addition of a 1-fold molar excess of poly(dT) to an ongoing helicase reaction resulted in a 51% decrease in DNA unwinding suggesting that the WRN helicase readily dissociates from the M13 partial duplex substrate and is not very processive (Fig. 7). To explore the mechanism of hRPA stimulation of WRN helicase activity, competition experiments were conducted in the presence of coating concentrations of hRPA. Even in the presence of hRPA, WRN helicase strand displacement was reduced by 37% at a 1-fold molar excess of poly(dT) (Fig. 7). These data show that hRPA did not significantly prevent WRN helicase from being efficiently competed from the helicase substrate.

**Immunoprecipitation**—Immunoprecipitation experiments were performed to investigate the possibility of a physical interaction between WRN and hRPA (Fig. 8). Single chain antibody fragments (scFv), selected against WRN from a phage display library, were coated on tosyl-activated magnetic beads as described under “Materials and Methods.” Two different monoclonal scFvs (N22 and WB19) were tested for their ability to coimmunoprecipitate WRN and hRPA from a solution of helicase reaction buffer containing hRPA and bovine serum albumin (BSA) with or without WRN, each at a concentration of 5 \( \mu \text{g/ml} \). After binding protein complexes to WRN antibody-coated beads, the beads were extensively washed in helicase reaction buffer containing 150 mM NaCl and 0.1% BSA. Western blot analysis of immunoprecipitated protein demonstrated that hRPA was precipitated by either N22 or WB19 scFv WRN monoclonal antibody (Fig. 8A). Immunoprecipitation of hRPA was dependent on the presence of WRN protein (Fig. 8B). The WRN-hRPA complex is resistant to dissociation during extensive washing in the presence of 150 mM NaCl and a 1000-fold molar excess of BSA, indicating a fairly specific and stable physical interaction between WRN and hRPA.
The precise molecular-genetic roles of the WRN gene product remain to be defined and characterized. The helicase activity associated with WRN protein indicates that the WS cellular phenotypes arise from deficiencies in some aspect of DNA metabolism. To understand better the functional roles of WRN protein, we have investigated the unwinding properties of the enzyme and the effect of hRPA on its activity. RPA has been extensively characterized and shown to be required in the processes of replication, recombination, and repair and may also be involved in transcription. Our results suggest that WRN and hRPA are likely to function together to perform one of these fundamental processes.

In this study, we have confirmed and significantly extended the initial observation of Shen et al. (28) that the ability of WRN helicase to unwind DNA duplexes of 42 bp is stimulated by the presence of SSB. WRN helicase can unwind a short DNA duplex (28 bp) in the absence of an SSB. However, an SSB is required for efficient unwinding of a slightly longer 69-bp partial duplex DNA substrate. An analysis of the requirement for an SSB to unwind the 69-bp duplex clearly shows that hRPA performed significantly better in this capacity than ESSB or gp32. These results are consistent with a previous study reporting that WRN helicase activity on a 42-bp partial duplex DNA substrate was stimulated better by hRPA than heterologous SSBs from E. coli or bacteriophage T4 (24). It has been postulated that SSB serves to prevent reannealing of unwound stands, thereby increasing the amount of free oligonucleotide product in a helicase reaction. However, the superior ability of hRPA over ESSB or gp32 to support WRN helicase activity suggests that hRPA performs an additional role in the WRN-catalyzed unwinding reaction other than simply coating the single strands generated during DNA duplex unwinding.

A specific interaction between WRN helicase and hRPA is
further supported by the absolute requirement for hRPA in the WRN-catalyzed unwinding of long DNA duplexes. Results from helicase assays using 257- and 849-bp partial duplex DNA substrates clearly show that hRPA is capable of facilitating the unwinding reaction. In contrast, ESSB (46–1456 nM, homotetramer) or gp32 (0.03–6042 nM, monomer) completely failed to stimulate WRN-catalyzed unwinding of these substrates. These data indicate that WRN helicase and hRPA coordinately unwind long DNA duplexes in vitro and suggest that the two proteins may functionally interact in vivo. The functional roles of helicases can be classified based on the length of the DNA duplex that each enzyme is capable of unwinding. Many helicases exhibit a limited reaction mechanism in which the fraction of DNA duplex molecules unwound decreases substantially as the duplex length increases (43). This is evident for the WRN helicase that alone efficiently unwinds the 28-bp partial duplex yet fails to unwind the 69- or 257-bp partial duplex substrates. The limited unwinding reaction of WRN helicase is overcome by the participation of an SSB. Previously, the longest DNA duplex reported to be unwound by WRN helicase was...
53 bp (22). Here we show that WRN helicase can unwind a tract of 849-bp DNA duplex in a reaction dependent on hRPA. Thus one role of hRPA in vivo may be to stimulate WRN helicase-catalyzed unwinding of long DNA duplexes.

SSBs have been shown to stimulate the activity of DNA helicases from yeast (45), calf thymus (46, 47), and human cells (48, 49). For example, the unwinding reactions of two human DNA helicases α and ε are stimulated by SSBs. Helicase α, isolated from HeLa cells, functioned much more efficiently in the presence or absence of ESSB compared with hRPA (49). Helicase activity on a 52-bp partial duplex DNA substrate was extremely poor (<5% substrate unwound) in the presence of hRPA, whereas ESSB significantly stimulated unwinding (~80% substrate unwound). A 5′-tailed substrate was unwound slightly better by helicase α in the presence of hRPA but not nearly as well as the ESSB-supported reaction. A nontailed partial duplex DNA substrate (~500 bp in length) was only weakly unwound by helicase α in a reaction completely dependent on ESSB. The 5′-tailed long partial duplex DNA substrate was more robustly unwound by helicase α in a reaction also dependent on ESSB. In contrast, WRN helicase can readily unwind long DNA duplexes lacking 5′ or 3′ tails, and its activity is stimulated by hRPA to a much greater degree than ESSB. Thus, the characteristics of the unwinding reaction catalyzed by helicase α are distinct from WRN helicase. Moreover, the functional interaction of WRN helicase with hRPA is not a universal property of all human DNA helicases.

Another DNA helicase activity isolated from HeLa cells, designated helicase ε, was shown to be efficiently stimulated by hRPA on a 30-bp partial duplex DNA substrate (48). The unwinding activity of helicase ε with hRPA was found to be 15-fold greater than with ESSB, whereas T4 gp32 completely failed to support unwinding. Helicase ε was also reported to unwind a 322-bp blunt duplex DNA fragment in a reaction dependent on hRPA. The peak of DNA helicase activity comigrated with a 72-kDa polypeptide detected on an SDS-polyacrylamide gel, distinct from the molecular weight of WRN protein. Sequence comparison between WRN protein and helicase ε revealed two probable interaction domains for hRPA will have to await the cloning of the gene encoding helicase ε. Helicase ε alone very poorly unwound a 30-bp partial duplex DNA substrate (~5% substrate unwound in 1 h) in contrast to the efficient unwinding reaction catalyzed by WRN protein alone on a 28-bp partial duplex DNA substrate. The fact that WRN helicase alone can efficiently unwind short DNA duplexes suggests that WRN helicase, unlike helicase ε, may function in hRPA-independent as well as hRPA-dependent pathways.

Specific stimulation of WRN helicase activity by hRPA suggests that the two proteins functionally interact. A possible explanation for the stimulation of WRN helicase activity by hRPA is that the enzyme either during translocation of the enzyme to the ssDNA-dsDNA junction or during the strand separation phase of the helicase reaction itself. The herpes simplex virus type 1 SSB, ICP8, has been recently shown to increase the processivity of the UL9 DNA

![Figure 7](http://www.jbc.org/) Effect of increasing concentration of competitor DNA on the unwinding of a 28-bp partial duplex substrate in the presence or absence of hRPA. Helicase reactions with the 28-bp partial duplex (2 μM nucleotide phosphate) were initiated with the addition of WRN protein (92 nM, heterotrimer) or WRN protein (92 nM, heterotrimer) plus hRPA (91 nM, heterotrimer). After 5 min of incubation at 24 °C, poly(dT) was added at the indicated concentrations. After 15 more min of incubation, reactions were quenched, and DNA helicase activity was determined. Quantitation of results are shown as follows: ●, hRPA; ○, no hRPA. Percent displacement is expressed as a function of fold molar excess of DNA competitor.

![Figure 8](http://www.jbc.org/) Coimmunoprecipitation of hRPA and WRN. Experiments using affinity beads coated with WRN monoclonal antibodies to immunoprecipitate WRN and hRPA were performed as described under "Materials and Methods." Proteins from immunoprecipitates were analyzed by Western blot. A, the membrane was probed with an antibody against the 70-kDa subunit of hRPA. Lanes 1 and 2 were precipitated with scFv N22-coated beads, and lanes 3 and 4 were precipitated with scFv WB19-coated beads. The protein mixtures used for lanes 1 and 3 included both WRN and hRPA, whereas only hRPA was present in lanes 2 and 4. B, the same blot was probed with an antibody against the WRN protein to demonstrate the precipitation of WRN (lanes 1 and 3).
helicase (50). It is generally believed that helicases utilize ATP hydrolysis to fuel translocation along the ssDNA lattice, although movement of WRN helicase along ssDNA has not been previously shown. We observed that hRPA failed to stimulate ssDNA-dependent ATPase activity of WRN protein raising the possibility that the tracking of WRN helicase along ssDNA was not rendered more processive. In DNA competitor experiments, we observed similar inhibition of WRN helicase activity in the presence or absence of hRPA suggesting that hRPA does not prevent the WRN helicase from being efficiently competed from the substrate. Taken together with the ATPase data, these results suggest that hRPA does not increase the processivity of WRN on the partial duplex DNA substrate.

It was also observed that the ssDNA-stimulated ATPase activity of human helicase ε was not affected by hRPA (48), despite the stimulatory effect of hRPA on the helicase activity of the enzyme. The observation that elongation length critically affects the hRPA requirement for WRN helicase activity suggests that hRPA plays a role in the elongation phase of the unwinding reaction as opposed to initiation. Conceivably, hRPA may facilitate productive binding of WRN helicase to the ssDNA-dsDNA junction of the ongoing helicase reaction. Alternatively, hRPA may prevent strand switching of the WRN helicase during unwinding by tethering the enzyme to one strand via protein-protein interactions. More mechanistic studies will be required to understand better the nature of hRPA stimulation of WRN helicase activity.

A functional interaction between WRN and hRPA is further supported by our demonstration of a physical interaction between the two proteins. Immunoprecipitation experiments indicate that WRN-specific monoclonal antibodies precipitate hRPA only in the presence of WRN protein. These data provide the first direct evidence that WRN and hRPA proteins physically interact with each other. The physical interaction between WRN and hRPA presumably mediates the specific stimulatory effect of hRPA on WRN helicase activity. In addition, hRPA may also modulate the exonuclease activity of WRN recently shown (26–28). This is the first demonstration of a physical/functional interaction of WRN with any other protein and raises the possibility that WRN may interact with other molecular partners in pathways involving hRPA.

The demonstration of a physical and functional interaction between WRN and hRPA suggests that the two proteins function together in some aspect of DNA metabolism in vivo. Genomic instability in WS may suggest a role for a WRN-hRPA complex in recombination or repair. Notably, WRN helicase expressed in S. cerevisiae suppresses hyper-recombination in an sgs1 mutant suggesting that WRN regulates genomic instability through suppression of recombination in human cells (15). Although RPA has been shown to stimulate the in vitro strand-exchange reaction (37, 38), a coordinate role of WRN and hRPA in the suppression of recombination remains to be shown. A dual role of WRN helicase in recombination is suggested by biochemical studies demonstrating that the bacterial WRN homolog, RecQ, acts in conjunction with RecA and ESSB proteins to initiate homologous recombination as well as disrupt joint molecules formed by aberrant recombination (51). In nucleotide excision repair, RPA has roles in damage recognition (31) and excision (32) as well as repair synthesis (33–35). Significantly, RPA-protein interactions are essential for the assembly of the excision complex. Although WRN has not been directly implicated in nucleotide excision repair, two observations have suggested that WRN protein may play a role in DNA repair as follows: 1) some WS cells are defective in transcription-coupled repair (19), and 2) WS cells exhibit a hypersensitivity to the “UV mimetic” agent 4-nitroquinoline 1-oxide (16, 17). Further studies will be necessary to evaluate a direct role for WRN helicase in recombination or repair.

The requirement of RPA for replication of chromosomal DNA supports the notion that WRN may be directly involved in replication initiation and/or elongation. Most recently, the Xenopus laevis replication focus-forming activity 1 (FFA1) was characterized as a DNA helicase and shown to exhibit extensive sequence homology to human WRN (52). Importantly, unlike a number of DNA helicases or putative helicases belonging to the RecQ family, the homology between WRN and FFA1 exists throughout the entire protein sequence suggesting that FFA1 is the true X. laevis functional homolog of human WRN. The assembly of RPA-containing replication foci in X. laevis egg extracts requires FFA1 (53). FFA1 binds to nuclear chromatin in an early stage of formation of functional replication origin complexes and enables RPA to subsequently bind to the foci. The recruitment of RPA to replication foci may be dependent on protein-DNA as well as protein-protein interactions. Thus, WRN, like FFA1, is a good candidate for the recruitment of RPA to replication foci via direct interaction with RPA as well as providing suitable ssDNA-binding sites for RPA. This notion is supported by the physical interaction between WRN and hRPA we have demonstrated in this study.

Aside from initiation, WRN helicase may function during replication elongation. RPA is required for elongation of DNA synthesis (29), but its role is not well understood. The fact that hRPA can stimulate WRN helicase to unwind hundreds of base pairs in vitro suggests that WRN helicase may function in some capacity as the replication fork processes. These hypotheses could be further explored using an in vitro replication system to evaluate a role of WRN in replication. A number of phenotypes of WS cells grown in culture are consistent with the notion that WRN helicase may play a direct role in replication. Genomic instability in WS cells, characterized by extensive deletions and chromosomal rearrangements, may arise due to basic defects in some aspect of DNA replication. The ability of WRN helicase to unwind long DNA duplexes is likely to be an important function in an essential pathway of DNA metabolism such as replication or recombination. Further studies characterizing the enzymatic activities and protein-protein interactions of WRN should help to elucidate the function of the protein and the pathway defective in WS.

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Interaction between WRN and hRPA

Functional and Physical Interaction between WRN Helicase and Human Replication Protein A
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