Werner syndrome is a hereditary premature aging disorder characterized by genomic instability. Genetic analysis and protein interaction studies indicate that the defective gene product (WRN) may play an important role in DNA replication, recombination, and repair. DNA polymerase β (pol β) is a central participant in both short and long-patch base excision repair (BER) pathways, which function to process most spontaneous, alkylated, and oxidative DNA damage. We report here a physical interaction between WRN and pol β, and using purified proteins reconstitute a portion of the long-patch BER pathway to examine a potential role for WRN in this repair response. We demonstrate that WRN stimulates pol β strand displacement DNA synthesis and that this stimulation is dependent on the helicase activity of WRN. In addition, a truncated WRN protein, containing primarily the helicase domain, retains helicase activity and is sufficient to mediate the stimulation of pol β. The WRN helicase also unwinds a BER substrate, providing evidence that WRN plays a role in unwinding DNA repair intermediates. Based on these findings, we propose a novel mechanism by which WRN may mediate pol β-directed long-patch BER.

Werner syndrome (WS)1 is a rare, autosomal recessive premature aging disorder characterized by gray hair, wrinkled skin, cataracts, atherosclerosis, osteoporosis, diabetes mellitus (type 2), and an increased incidence of malignancies (1). The gene (WRN) defective in WS patients encodes a 1,432-amino acid protein that contains ATnPase, 3′ → 5′ helicase, and 3′ → 5′ exonuclease activities (reviewed in Ref. 2). Cells from WS patients display an extended S phase (3), undergo premature replicative senescence (4), and exhibit higher levels of DNA deletions, translocations, and chromosomal breaks (5, 6). The increased genomic instability associated with WS cells suggests that WRN may function in DNA replication, recombination, and/or repair pathways. Sensitivity of WS cells to DNA-damaging agents provides additional evidence for a role of WRN in DNA repair. WS cells show marked hypersensitivity to 4-nitroquinoline 1-oxide (4-NQO), topoisomerase inhibitors, and DNA interstrand cross-linking agents (reviewed in Ref. 2). In addition, a mild sensitivity of WS cells to ionizing radiation (IR) has been demonstrated (7, 8). In contrast, WS cells display no or little sensitivity to ultraviolet light, bleomycin, hydroxyurea, or alkylating agents (reviewed in Ref. 19). However, a recent report shows that WRN–/– chickens are hypersensitive to the alkylating agent methyl methanesulfonate (MMS) (10). 4-NQO generates a wide spectrum of DNA adducts as well as oxidative damage (11, 12), and IR produces a diverse range of DNA damage including strand breaks and oxidative base modifications (13, 14). Hypersensitivity of WS cells to these damaging agents suggests that WRN may participate in some aspect of oxidative DNA damage repair.

Base excision repair (BER) is important for the repair of a wide variety of DNA damage including oxidized, alkylated, deaminated, and hydrolyzed bases (reviewed in Refs. 15 and 16). DNA glycosylases recognize damaged bases and cleave the glycosyl bond. The resulting apurinic/apyrimidinic (AP) site is then cleaved 5′ to the abasic site by an AP endonuclease (APE1), resulting in the formation of a 3′-hydroxyl and a 5′-deoxyribose phosphate (dRP). In mammalian cells, completion of BER occurs by short-patch (single nucleotide incorporation) or long-patch (replacement of 2–13 nucleotides) repair. In the short-patch pathway, DNA polymerase β (pol β) removes the dRP group and fills in the resulting gap, which is then sealed by a DNA ligase. If the dRP group is modified (most notably reduced) and refractory to removal by pol β dRP lyase activity, repair likely occurs through the long-patch pathway. In this alternative process, strand displacement and DNA synthesis by either pol β and/or pol δ replaces several nucleotides. The resulting 5′-flap is excised by flap endonuclease (FEN-1), and the nick is sealed by a DNA ligase. Long-patch BER catalyzed by pol δ ε also requires proliferating cell nuclear antigen (PCNA) and replication factor C (RFC) to enhance polymerase activity. PCNA has also been shown to stimulate FEN-1 flap cleavage (17). In addition, a number of accessory proteins have been reported to participate in and/or stimulate BER including poly(ADP-ribose) polymerase-1 (PARP-1) (reviewed in Ref. 18), p53 (19), and replication protein A (RPA) (20).

WRN has been shown to interact physically and/or functionally with several proteins involved in long-patch BER including pol δ, PCNA, RPA, and FEN-1. WRN interacts physically with pol δ and recruits it to the nucleolus (21). Furthermore, WRN has been shown to stimulate nucleotide incorporation by pol δ (22). A physical interaction between WRN and PCNA has been identified, however, a functional interaction has yet to be determined (23). RPA stimulates the WRN helicase to unwind long duplex DNA substrates that WRN alone is not able to...
unwind (24, 25). WRN interacts physically with FEN-1 and stimulates its DNA flap cleavage activity (26). However, because these proteins participate in both replication and long-patch BER, an association with WRN does not reveal whether this interaction is important for one or both pathways. To distinguish a role for WRN in DNA repair, we investigated a potential interaction between WRN and pol β.

We report here that WRN physically interacts with pol β. As protein-protein and protein-DNA interactions mediate the repair steps involved in BER, and pol β plays a central role in BER, we examined a potential functional role for this interaction by reconstituting a portion of the long-patch BER pathway. We find that pol β strand displacement synthesis is enhanced by WRN and this stimulation requires WRN helicase activity. Furthermore, a glutathione S-transferase (GST)-WRN fusion protein containing the helicase domain of WRN catalyzes unwinding and is sufficient to mediate the stimulation of pol β. We also demonstrate that WRN unwinds a BER intermediate suggesting a novel mechanism through which WRN may participate in pol β-mediated long-patch BER.

EXPERIMENTAL PROCEDURES

Materials

[α-32P]dATP, [α-32P]dCTP, ATP, and dNTPs were purchased from Amersham Biosciences. [γ-32P]ATP was purchased from PerkinElmer Life Sciences, polynucleotide kinase from New England BioLabs, and terminal deoxynucleotidyltransferase (TdT) from Promega.

Proteins

Purified recombinant proteins used in this study are presented in Fig. 1, A and B. Endonuclease (APE1) (27), pol β (28), uracil DNA glycosylase (UDG) (29), wild-type WRN (30), the WRN helicase mutant K577M (K-WRN) (30), and FEN-1 (26) were purified as described previously. Recombinant GST-WRN fusion proteins were overexpressed in Escherichia coli and purified using glutathione beads (Amersham Biosciences) as described previously (26). Bands migrating below GST-WRN (949–1432) (~75 kDa) were determined to be degradation products by Western blot analysis using anti-GST antibodies.

Cell Lysate Preparation and Co-immunoprecipitation

Telomerase-immortalized wild-type (WT, hTERT-GM01604), and Werner syndrome (WS, hTERT-AG03141) cell lines (a gift from Jerry Shay) were cultured in 150-mm dishes until nearly confluent. Cells were incubated in a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 25 mM NaF, 0.1 mM sodium orthovanadate, 0.2% Triton X-100, 0.3% Nonidet P-40) containing protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 5 μg/ml leupeptin) on ice for 30 min and then centrifuged at 14,000 rpm for 30 min at 4 °C. The protein concentration was determined using the Bio-Rad protein assay, with bovine serum albumin (BSA) as a standard. For co-immunoprecipitation, an equal amount (1 mg of protein) of cell lysate was mixed with 0.7 μg of affinity-purified anti-pol β polyclonal antibody, anti-WRN polyclonal antibody (SC-9469, Santa Cruz Biotechnology), or rabbit non-immune IgG, respectively. The mixture was incubated with rotation for 4 h at 4 °C. The protein-antibody immunocomplexes were absorbed onto protein A-Sepharose/protein G-agarose beads. The proteins were eluted in sodium dodecyl sulfate (SDS) sample buffer, separated by 4–12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and immunoblotted using an anti-WRN monoclonal antibody (1:200 dilution, BD Transduction Laboratories). The same blot was stripped by incubating with buffer containing 62.5 mM Tris-HCl, pH 6.8, 100 mM β-mercaptoethanol, and 1% SDS for 30 min at 50 °C, followed by Western analysis using anti-pol β monoclonal antibody, 18 S (31).

GST-WRN Pull-down Assay

HeLa nuclear extracts (NE) pull-down assays with GST and a GST-C-terminal WRN fragment (GST-WRN949–1432) with HeLa NE were performed essentially as described elsewhere (26, 32). Briefly, GST or the GST-WRN fragment was bound to glutathione beads and then incubated with HeLa NE (~1 mg). After washing, proteins were eluted in sample buffer by boiling and electrophoresed on 10% polyacrylamide SDS gels and transferred to polyvinylidene difluoride membranes. GST-C-terminal WRN fragment and bound proteins were detected by Amido Black staining of the membrane. pol β bound to GST-WRN (949–1432) was detected by Western blot with mouse monoclonal anti-pol β antibodies (Trevenig, 1:5000).

Mapping Using Purified pol β

GST or GST-WRN fragments were prebound to glutathione beads and then incubated with purified pol β (5 μg) for 1.5 h at 4 °C. Following several washes with phosphate-buffered saline (1×) and 0.1% Tween 20, bound proteins were resuspended in sample buffer and analyzed by Western blot as described above.

Co-immunoprecipitation with Purified Proteins

Co-immunoprecipitation experiments were performed essentially as described previously (33). Briefly, purified WRN (1.5 μg) and pol β (1.5 μg)
Enzyme-linked Immunosorbonent Assay (ELISA)

The detection of protein interactions was performed by ELISA essentially as described previously (35). Wells were coated with pol β (75 ng) or BSA (negative control) for 16 h at 4 °C. Wells were washed and incubated with blocking buffer (phosphate-buffered saline, 2% BSA, 0.1% Tween 20) for 1 h at 37 °C. Following washing, primary antibody (anti-rabbit pol β, Novus, 1:10,000) was added and incubated for 1 h at 37 °C. Wells were washed and secondary antibody (anti-rabbit IgG-horse radish peroxidase, Vector, 1:10,000) was added and incubated for 1 h at 37 °C. Bound WRN was detected using o-phenylenediamine dihydrochloride (OPD) as a substrate. The reaction was terminated by the addition of 3 M H₂SO₄. Absorbance was read at 490 nm. For the ethidium bromide controls, ethidium bromide was added to the binding step at a concentration of 10 μg/ml.

Helicase Assay

Forked Substrate—Helicase activity was detected by the displacement of a 32P-labeled 5′ oligonucleotide (5′-T₃CATGAGTGTTG -GTACATGCACTAC-3′) from its partial duplex with the complementary unlabeled oligonucleotide (5′-GTACATGCACTACCCACACT-C(T₃)₂-3′). Reactions were performed as described previously (36). Briefly, 5′ end-labeled DNA substrate (0.5 nm) was incubated in helicase reaction buffer (40 mM Tris-HCl, pH 8.0, 4 mM MgCl₂, 5 mM dithiothreitol (DTT), 2 mM ATP, and 0.1 mg/ml BSA) in a final volume of 20 μl. Reactions were initiated by the addition of WRN, incubated at 37 °C for 15 min, and terminated by the addition of 3× stop dye to a final concentration of 1× along with a 10× molar excess of unlabeled competitor oligonucleotide. Products were run on a 12% native polyacrylamide gel, visualized using a PhosphorImager, and quantitated using Image-Quant software.

Uracil-containing Substrate—The 34-mer uracil-containing DNA end-labeled with terminal deoxynucleotidyl transferase (TdT) and [α-32P]dATP. Prior to the helicase reaction, the substrate (20 nM) was preincubated with 2 mM UDG in 50 mM HEPES, pH 7.5, 20 mM KCl, and 2 mM DTT for 20 min at 37 °C. Helicase reactions (10 μl) contained 5 mM pretreated DNA substrate in helicase reaction buffer, with WRN, pol β(FEN-1), and dNTPs as indicated. Reactions were initiated by adding APEI and incubated for 25 min at 37 °C. Reactions were terminated and analyzed as described above.

Exonuclease Assay

Exonuclease activity was detected by the degradation of a 32P-labeled 5′ oligonucleotide (oligo 5), which was annealed to an unlabeled partial duplex complementary oligonucleotide (oligo 6) as described (36). Reactions were performed as described previously (36). Briefly, 5′ end-labeled DNA substrate (0.5 nm) was incubated in a standard reaction containing 40 mM Tris-HCl, pH 8.0, 4 mM MgCl₂, 5 mM DTT, 2 mM ATP, and 0.1 mg/ml BSA in a final volume of 10 μl. Reactions were initiated by the addition of WRN and WRN and incubated for 15 min at 37 °C. Reactions were terminated by the addition of an equal volume of formamide stop dye (80% formamide, 0.5× Tris borate/EDTA buffer, 0.1% bromophenol blue, and 0.1% xylene cyanol). Products were heat-denatured at 90 °C for 5 min and run on 14% denaturing polyacrylamide gels. Radioactive products were visualized using a PhosphorImager.

In Vitro BER Assay

Reconstitution of the repair reaction was performed essentially as described previously (37). Prior to assembly of the repair reaction, uracil-containing DNA substrate (400 nm) was preincubated with 400 mM UDG in 50 mM HEPES, pH 7.5, 20 mM KCl, and 2 mM DTT for 20 min at 37 °C (Fig. 5A). Repair reactions (10 μl) contained 100 mM pretreated DNA substrate in 50 mM HEPES, pH 7.5, 20 mM KCl, 2 mM DTT, 10 mM MgCl₂, 4 mM ATP (except where noted), 20 μM each of dATP, dGTP,
dTTP, 2 μM dCTP, 2.3 μM [α-32P]dCTP, and UDG (10 nM). The presence of WRN, K-WRN, GST-WRN500-946, or FEN-1 is indicated in the figure legends. Reactions were initiated by the addition of 10 nM APE1 and 5 nM pol β. The reaction mixture was incubated for 25 min at 37°C and terminated by the addition of an equal volume of stop dye (95% formamide, 20 mM EDTA, 0.02% bromphenol blue, and 0.02% xylene cyanol). Following incubation at 95°C for 5 min, the reaction products were separated by electrophoresis in a 20% denaturing polyacrylamide gel and visualized using a PhosphorImager. Under these reaction conditions, the incised abasic site produced by the sequential actions of UDG and APE1 was determined to be 90% (data not shown). Percent of total nucleotides incorporated was calculated as: % of total (amount of radioactivity associated with each dNMP addition/total radioactivity) × 100.

**pol β Primer Extension Assay**

Reactions (10 μl) contained 50 mM HEPES, pH 7.5, 20 mM KCl, 2 mM DTT, 10 mM MgCl₂, 1 mM each dATP, dGTP, dTTP, dCTP, and pol β and WRN as indicated in the figure legend. The reaction was initiated by the addition of 5 nM 5' end-labeled 15-mer (5'-CTGCAGCTGATGCGC-3') annealed to a 34-mer unlabeled complementary oligonucleotide (5'-GTACCAGGCCGTACGGCGCATACGCTGAG-3') and incubated at 37°C for 15 or 30 min. The reaction mixture was terminated by the addition of an equal volume of stop dye (95% formamide, 20 mM EDTA, 0.02% bromphenol blue, and 0.02% xylene cyanol). Reaction products were incubated at 90°C for 5 min, run on 20% denaturing polyacrylamide gels, and visualized using a PhosphorImager.

**RESULTS**

Physical Interaction and Complex Formation between WRN and pol β—To investigate a potential role for WRN in the repair of oxidative DNA damage, we first performed co-immunoprecipitation assays to identify an interaction between WRN and pol β. Using antibodies against pol β, we co-immunoprecipitated WRN from normal (Fig. 2A, lane 1) but not WS cells (Fig. 3A). WRN and pol β physically interact. A, recombinant WRN or K-WRN were incubated in the presence or absence of pol β as indicated and immunoprecipitated with antibodies against pol β (lanes 1–3) or preimmune IgG (lane 4). Lanes 5 and 6, purified pol β (50 ng) and WRN (50 ng), respectively. Upper panel, immunoblotting with anti-WRN monoclonal antibody. Lower panel, the blot was stripped and re-probed with anti-pol β monoclonal antibody. B, purified pol β was incubated in the presence of WRN or K-WRN as indicated and immunoprecipitated with antibodies against WRN (lanes 1–3) or preimmune IgG (lane 4). Lanes 5 and 6, purified WRN (60 ng) and pol β (50 ng), respectively. Upper panel, immunoblotting with anti-pol β monoclonal antibody. Lower panel, the blot was stripped and re-probed with anti-WRN monoclonal antibody. C, either BSA (bars 1 and 2) or purified recombinant pol β (bars 3–5) was coated onto ELISA plates. After blocking with BSA, the wells were incubated with BSA (bars 1 and 3), purified WRN (bars 2 and 4), or ethidium bromide (10 μg/ml) plus purified WRN (bar 5). Bound WRN was detected using anti-WRN antibodies and horseradish peroxidase-labeled secondary antibodies. The values represent with mean of at least three independent experiments performed in duplicate. D, GST alone (lane 1) or recombinant GST-WRN fragments (lanes 2–4, as indicated) were bound to glutathione beads and incubated with purified pol β. After washing, bound pol β was determined by SDS-PAGE and Western blot analysis. Purified pol β (10 ng) was loaded as a marker (lane 5). Amido Black staining shows the amount of GST-WRN fragments loaded.
We also co-precipitated pol β/H9252 from wild-type cells using antibodies against WRN (Fig. 2B, lane 1) but not from WS cells (Fig. 2B, lane 2). As the C terminus of WRN is important for mediating interactions with many of its protein partners, including pol δ (21), we used a pull-down assay with WRN and FEN-1 stimulate strand displacement DNA synthesis by pol β. A, schematic representing the 34-bp DNA substrate containing a uracil at position 16 prior to and following treatment with UDG (40 nM) for 20 min at 37 °C. B, the UDG-pretreated DNA (100 nM) was then incubated for 25 min at 37 °C in the presence (+) or absence (−) of pol β, FEN-1, or WRN as indicated. All reaction mixtures contained APE1 (10 nM) and UDG (10 nM). C, quantitation of short (1) and long-patch (2–6) BER intermediates. D, quantitation of individual long-patch BER intermediates. Percent of total nucleotides incorporated was calculated as described under “Experimental Procedures.” Values represent the mean and S.D. of four independent experiments.
a C-terminal WRN fragment (GST- WRN<sub>949–1432</sub>) to investigate a possible association between WRN and pol β in HeLa nuclear extracts. pol β co-precipitated with GST-WRN<sub>949–1432</sub> (Fig. 2C, right panel, lane 4). This association with GST-WRN<sub>949–1432</sub> was specific as GST alone did not precipitate pol β (Fig. 2C, right panel, lane 3). These results suggest that WRN and pol β are in the same complex in vivo.

To test whether WRN and pol β physically interact, we performed co-immunoprecipitation experiments with purified, recombinant proteins. As shown in Fig. 3A, WRN and K-WRN (a WRN ATPase/helicase mutant, Fig. 1A) were precipitated by pol β antibodies (Fig. 3A, lanes 1 and 2), but not when pol β was omitted (Fig. 3A, lane 3) or when the immunoprecipitation was performed with preimmune IgG (Fig. 3A, lane 4). When WRN antibodies were used for the co-immunoprecipitation experiments, pol β was effectively precipitated (Fig. 3B, lanes 1 and 2). However, when WRN was omitted (Fig. 3B, lane 3) or preimmune IgG was used (Fig. 3B, lane 4) pol β was not detected. To further confirm a direct association between WRN and pol β, we performed ELISA with purified proteins. pol β specifically bound to WRN and not to the BSA control (Fig. 3C). This interaction was not mediated by DNA since the binding was not affected by the presence of ethidium bromide (Fig. 3C).

The Effect of pol β on WRN Activities—Based on the physical interaction between WRN and pol β, we next tested whether pol β functionally altered WRN helicase or exonuclease activities. On a forked 22-bp duplex DNA substrate, increasing concentrations of pol β did not influence WRN helicase activity (Fig. 4A). Similarly, WRN exonuclease activity on a forked oligonucleotide containing a 34-bp duplex was not affected by pol β (Fig. 4B). These results suggest that under the reaction conditions employed, pol β does not modulate WRN helicase or exonuclease activities.

WRN Stimulates pol β DNA Strand Displacement Synthesis—To determine whether WRN influences pol β-mediated long-patch BER, we reconstituted a portion of the long-patch BER reaction using a 34-bp oligonucleotide substrate containing a uracil lesion at position 16 and the following purified human enzymes: UDG, APE1, pol β, and FEN-1 (Fig. 5A). Without the addition of DNA ligase, 16-mer oligonucleotide products represent incorporation of a single [α-<sup>32</sup>P]dCMP nucleotide and short-patch BER. Longer products represent strand displacement DNA synthesis and long-patch BER intermediates. pol β synthesizes primarily the 16-nucleotide product (Fig. 5B, lane 1), which represents one-nucleotide gap-filling. The addition of FEN-1 produced a modest stimulation in strand displacement synthesis (Fig. 5B, lane 2) as has been published previously (37). When WRN was added, a comparatively greater stimulation of pol β strand displacement synthesis was seen (Fig. 5B, lane 3). When FEN-1 and WRN were added together, the greatest stimulation resulted (Fig. 5B, lane 4). These results suggest that WRN, particularly when present with FEN-1 in this in vitro system, produces a strong stimulation of strand displacement synthesis by pol β.

Consistent with the above results, quantitation of the number of nucleotides incorporated by pol β alone indicated primarily short-patch BER intermediates (90% of the synthesis, Fig. 5C). The presence of either FEN-1 or WRN alone decreased the number of short-patch intermediates to ~70%, while increasing the long-patch intermediates to ~30% (Fig. 5C). The addition of both FEN-1 and WRN resulted in ~55% of long-patch intermediates (Fig. 5C). Furthermore, FEN-1 stimulated predominantly long-patch intermediates at the 2 nucleotide position (~17%) while addition of WRN produced mostly 4 nucleotide products (~13%) (Fig. 5D). WRN also increased pol β-mediated strand displacement DNA products to 5 and 6 nucleotides in length (Fig. 5D). Since long-patch BER typically involves 2–13 nucleotide repair patches, these results clearly demonstrate that WRN, in both the absence and presence of FEN-1, stimulates the synthesis of long-patch BER intermediates by pol β.

WRN Stimulation of pol β Requires a BER Substrate—To further examine the stimulation of pol β strand displacement synthesis by WRN, we tested the influence of WRN on nucleotide incorporation by pol β. We used a 15-mer primer, which represents removal of the downstream 18-bp oligonucleotide produced following UDG and APE1 treatment of the original 34-bp uracil-containing DNA substrate. Reactions to determine pol β processivity in the presence of WRN were performed under single hit conditions and primer extension was limited to less than 20% as defined previously (38). Single hit conditions refer to extension of a primer via interaction with a DNA polymerase at most once, followed by polymerase dissociation. As shown in Fig. 6, WRN did not affect the ability of pol β to incorporate nucleotides from this primer/template. Consistent with our findings, a previous report by Kamath-Loeb et al. (22) found that WRN did not affect DNA synthesis by pol β. Thus, the WRN-mediated stimulation of pol β strand displacement synthesis does not appear to be the result of WRN stimulating pol β DNA polymerization.

WRN Helicase Activity Stimulates pol β Strand Displacement Synthesis—To further characterize the stimulation of pol β strand displacement synthesis, we used a WRN variant (K-WRN) that contains a mutation in the ATPase domain (K577M) which abolishes helicase, but not exonuclease activity (24, 39). We also used a GST-WRN fusion protein containing amino acids 500–946 of WRN, which includes the helicase domain and excludes the exonuclease and extreme C-terminal domains (Fig. 1A). This helicase fragment has not previously

**Fig. 6. Polymerization by pol β on a primer/template is not influenced by WRN.** Where indicated, reaction mixtures contained pol β (0.0125 nM) and 0.0125 nM (lanes 4 and 9), 0.075 nM (lanes 5 and 10), or 0.15 nM (lanes 2, 6, 7, and 11) WRN. Reactions were initiated by the addition of the 5′-[<sup>32</sup>P]labeled 15-mer primer annealed to a 34-mer template (5 nM) and incubated at 37 °C for 15 (lanes 1–6) or 30 (lanes 7–12) min. Following heat denaturation at 90 °C for 5 min, products were analyzed on 20% denaturing polyacrylamide gels. The bold arrow indicates the position of the 15-mer primer. The smaller arrow indicates the 14-mer product generated by WRN exonuclease activity.
Fig. 7. Stimulation of pol β strand displacement synthesis by WRN is dependent on its helicase activity. A, reactions contained GST as a negative control (1.5 μM, lane 3), 0.125, 0.25, 0.5, and 1 nM WRN (lanes 4–7, respectively) or 16, 32, 65, and 130 nM GST-WRN<sub>500-946</sub> (lanes 8–11, respectively). Proteins were incubated with a 22-bp forked duplex (0.5 μM) for 15 min at 37 °C. Lane 2, WRN protein storage buffer; lane 12, heat-denatured substrate control. Products were run on a 12% native gel and visualized using a PhosphorImager. B, amount of single-stranded DNA (Fig. 7A). GST on a forked substrate containing a 22-bp duplex (Fig. 7A). GST-WRN (lanes 2–4, respectively), K-WRN (7.5, 15, and 30 nM; lanes 5–7, respectively), or GST-WRN<sub>500-946</sub> (0.2, 0.4, and 0.6 μM; lanes 8–10, respectively) as indicated. All reaction mixtures contained APE1 (10 nM) and UDG (10 nM). C, the UDG-pretreated DNA (100 nM) was incubated for 25 min at 37 °C with pol β (5 nM) and increasing concentrations of WRN (7.5, 15, and 30 nM; lanes 2–4, respectively), or GST-WRN<sub>500-946</sub> (0.4 μM) as indicated. All reaction mixtures contained APE1 (10 nM) and UDG (10 nM). Lane 2, GST (3 μM) negative control. D, the UDG-pretreated DNA (100 nM) was incubated with pol β, WRN, or FEN-1 as indicated in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of ATP. All reaction mixtures contained APE1 (10 nM) and UDG (10 nM).

been tested biochemically. To investigate whether the GST-WRN<sub>500-946</sub> fragment could function as a helicase, we examined the unwinding activity of the GST-WRN helicase fragment on a forked substrate containing a 22-bp duplex (Fig. 7A). GST alone had no effect (Fig. 7A, lane 3). Increasing amounts of wild-type WRN resulted in an increase in the amount of labeled strand displaced (Fig. 7A, lanes 4–7). Similarly, increasing concentrations of GST-WRN<sub>500-946</sub> produced increasing amounts of the unwound strand (Fig. 7A, lanes 8–11). Thus, the GST-WRN fusion protein containing primarily the helicase domain of WRN unwinds a forked duplex as does wild-type WRN. To our knowledge, this is the first demonstration that the helicase domain of WRN alone can function as an active helicase.

Using wild-type WRN, K-WRN, and GST-WRN<sub>500-946</sub> proteins, we examined the contribution of WRN helicase activity on the stimulation of pol β strand displacement synthesis. Our results show that wild-type WRN stimulates pol β strand displacement synthesis in a concentration dependent manner (Fig. 7B, lanes 2–4). However, the stimulation seen by wild-type WRN is not observed with K-WRN (Fig. 7B, lanes 5–7).

Conversely, the addition of increasing amounts of the WRN helicase fragment resulted in the greatest stimulation of pol β strand displacement synthesis (Fig. 7B, lanes 8–10). The relative amount of GST-WRN<sub>500-946</sub> chosen produced similar unwinding on a forked duplex, but resulted in greater pol β strand displacement synthesis compared with wild-type WRN. As demonstrated in Fig. 3D, pol β binds to the helicase domain of WRN (aa 500–946), and the physical interaction between this region of WRN and pol β may be important for the stimulation of strand displacement synthesis.

As wild-type WRN contains both helicase and exonuclease activity, while K-WRN contains only exonuclease activity, we next determined the contribution of a WRN protein which only has helicase activity. We tested a WRN variant (X-WRN) containing a point mutation in the exonuclease domain (E84A), and as shown in Fig. 7C, increasing concentrations of X-WRN resulted in a concentration-dependent stimulation of pol β strand displacement synthesis.

The presence of FEN-1 enhanced the wild-type WRN stimulation of pol β strand displacement synthesis (Fig. 7D, lane 5), but had no effect on K-WRN (Fig. 7D, lane 7). The addition of
FEN-1 to GST-WRN500-946 did not result in any additional stimulation (Fig. 7D, lanes 8 and 9). Perhaps the dramatic stimulation produced by GST-WRN500-946 alone precluded any additional stimulation by FEN-1. It is also possible that a WRN/FEN-1 physical interaction is required for the synergistic stimulation. The WRN C terminus (aa 949–1432) has been shown to bind FEN-1 (26), however, it is not known whether GST-WRN500–946 is sufficient to mediate binding. Finally, the stimulation seen by GST-WRN500–946 was not due to GST, as GST alone did not affect pol β strand displacement synthesis (Fig. 7D, lane 2).

Since the helicase activity of the WRN protein is dependent on ATP hydrolysis, we performed the in vitro long-patch BER reaction in the presence and absence of exogenous ATP. Incorporation of nucleotides by pol β was similar in the presence and absence of ATP (Fig. 7E, lanes 1 and 2). In addition, the stimulation of pol β strand displacement synthesis by FEN-1 was not dependent on ATP (Fig. 7E, lanes 5 and 6). However, the WRN-mediated stimulation of strand displacement synthesis by pol β was enhanced in the presence of ATP (Fig. 7E, compare lanes 3 with 4, and 7 with 8) consistent with above observations that an active WRN helicase is necessary for the stimulation of pol β strand displacement DNA synthesis.

To determine whether the stimulation of strand displacement synthesis by WRN was specific, we also tested whether the Bloom syndrome protein (BLM), another member of the RecQ family of helicases, was also capable of mediating the stimulation. We tested equal molar concentrations of WRN and BLM together in the same experiments and found that WRN was more efficient than BLM in stimulating strand displacement synthesis by pol β (Table I). Furthermore, while increasing the concentration of WRN from 15 to 30 nM resulted in a concentration-dependent increase in strand displacement synthesis products, the BLM-mediated stimulation was similar at both 15 and 30 nM. Thus, although at least two members of the RecQ family of helicases are able to stimulate pol β strand displacement synthesis, the stimulation appears to be more specific for WRN.

**WRN Can Unwind a BER Substrate**—Finally, we addressed whether or not WRN could unwind the uracil-containing substrate following treatment with UDG and APE1. The 34-mer containing uracil was labeled at the 3′-end with [α-32P]dATP and TdT generating a 35-mer (and a 19-mer following UDG and APE1 treatment). As expected, in the absence of APE1, WRN was not able to unwind the 35-mer containing an AP site (Fig. 8, lanes 5 and 10) as WRN helicase activity on blunt-ended duplex substrates is poor (40). However, in the presence of APE1, the WRN protein unwound the 19-bp oligonucleotide containing a dRP, which was generated following incision of the abasic site by APE1 (Fig. 8, lanes 6 and 11). These results present a novel substrate for the WRN helicase: an oligonucleotide containing a nick with a 3′-OH and a 5′-dRP. With the addition of pol β, dNTPs, and FEN-1 to the reaction, WRN was still able to displace the 19-mer, however, to a lesser extent than pol β alone (25%, suggesting a role for WRN in pol β-mediated long-patch BER. This is the first report that a fragment comprising primarily the helicase domain only, the strand displacement synthesis products increased from 10% (pol β alone) to ~25%, suggesting a role for WRN in pol β-mediated long-patch BER. This is the first report that a fragment comprising primarily the helicase domain of WRN retains activity. In addition, full-length WRN and BLM unwind a BER strand break intermediate produced following UDG and APE1 treatment of a uracil containing oligonucleotide. Previous studies have shown that WRN inefficiently unwinds a nicked duplex (40, 41). Thus, a BER intermediate represents a new substrate for the RecQ helicases. Since the reactions contained both UDG and APE1, it remains to be determined whether WRN recognizes and unwinds the substrate in the absence of these BER proteins, or requires...
physical interaction with the proteins themselves or the protein-DNA complex as has been suggested for other BER proteins (42). Nonetheless, our results demonstrate that the WRN helicase can participate in long-patch BER together with pol β.

Previously, extracts from WS cells were shown to efficiently repair a plasmid containing an AP site (43). This was a general measure of BER, as AP sites are mainly repaired by short-patch BER, indicating that WS cells are proficient in this process. However, the ability of extracts from WS cells to repair lesions requiring long-patch BER in vitro remains to be investigated. In addition, it will be important to find DNA damaging agents that enrich for lesions specifically repaired by the long-patch BER pathway. Given the relatively mild phenotype of WS, it is likely that a deficiency in WRN confers only mild sensitivity to agents which cause oxidative damage. Because of the small percentage of lesions that are repaired by long-patch BER, it may be difficult to identify significant differences in sensitivities to oxidative DNA-damaging agents in WS cells. Furthermore, based on the importance of maintaining genomic integrity following insult by reactive oxygen species, there likely exist many backup processes in BER. It is also possible that other helicases, such as BLM, substitute in the absence of WRN. However, the sensitivity of WS cells to agents which produce oxidative DNA damage (4-NQO and IR), and the in vitro evidence presented here, suggest that WRN may participate in vivo to coordinate interactions between BER proteins and/or to facilitate strand displacement in general or under special circumstances (see below).

In support of the notion that WRN participates in the early steps of BER, progression of the WRN exonuclease is blocked by some oxidative DNA modifications such as AP sites, 8-oxoguanine, and 8-oxoadenine (44). Pausing at sites of oxidative lesions may be an early step in the DNA damage recognition process, and WRN may serve to recruit DNA repair proteins to the site of the lesion via protein-protein interactions. After the damage has been sensed, WRN may serve additional roles through its helicase and exonuclease activities.

Although WRN may not be an essential component of BER, it may play an important role during the repair of certain lesions during long-patch BER. For example, oxidized abasic residues may function as suicide substrates in the formation of protein-DNA cross-links, as has been demonstrated for 2-deoxyribonolactone residues and pol β (45). These cross-linked intermediates may not be readily repaired by short-patch BER and may require alternative modes of repair. WRN may function to unwind such intermediates and/or stimulate FEN-1 cleavage of these protein-DNA substrates.

DNA helicases promote the separation of complementary strands of the DNA duplex providing proteins access to the template during replication, recombination, and repair (46). Helicases have been shown to function in DNA repair pathways such as nucleotide excision repair (NER) and mismatch repair (MMR). In NER, two DNA helicases XPF and XPD (xeroderma pigmentosum complementation groups B and D, respectively) unwind the site around the DNA lesion (47). In the methyl-directed MMR pathway in E. coli, helicase II unwinds the DNA from the site of the nick generated at the mismatch on the newly synthesized strand (48). Evidence presented here supports a novel role for helicases in BER. A helicase is likely required to unwind alternative structures produced in BER, and WRN is a good candidate.

In addition, WRN has been shown to unwind alternate DNA structures including triplex DNA (49), Holliday junctions (50), and DNA tetraplexes (51). WRN also enables pol δ to synthesize past hairpin and tetraplex structures of the d(CCGG), trinucleotide repeat sequence. Furthermore, WRN helicase activity is essential to alleviate pausing of pol δ at these tetraplex regions (52). Triplets of bases repeated in tandem can form a variety of structures including single-strand hairpin loops which may result in slippage and expansion by DNA polymerases. Recently, Hartenstine et al. (53) reported that the weak strand displacement activity of pol β results in the expansion of CAG/CTG triplet repeats. They further speculate that weak strand displacement activity in DNA repair at strand breaks may enable short tracts of repeat sequences to be converted into longer, more mutable stretches associated with neurological diseases. As polymerase-initiated DNA synthesis errors most likely play a central role in human aging and disease, it is interesting to speculate that WRN may increase the fidelity of polymerases (such as pol β and pol δ) via unwinding of alternate structures or through stimulation of strand displacement DNA synthesis (pol β).

pol β does not have an intrinsic editing function, and thus makes frequent errors, at the level of 1 per 4,000 nucleotide incorporation events (16). Unless special processes exist to increase the accuracy of BER, error-prone repair may result in mutations and cancer. One possible mechanism for increasing the fidelity of repair is a separate editing enzyme, which would remove nucleotides misincorporated by pol β. WRN is a 3′ → 5′ exonuclease, and the sequence of the WRN exonuclease is homologous to the 3′ → 5′ proofreading domain of E. coli DNA polymerase I (54). Furthermore, WRN has been shown to remove 3′ mismatches (55, 56) and is one of several proteins, which has been suggested to serve this proofreading function (55, 57). It remains to be determined whether WRN increases the fidelity of pol β and/or serves as a proofreading enzyme for pol β during repair events. The physical and functional data presented here provide further support for a role of WRN, together with pol β during repair of oxidative lesions.

Acknowledgments—We thank Lale Dawut and Nicholas McCollum for technical assistance. We also thank Drs. Wen-Hsing Cheng and David M. Wilson III for helpful discussions and critical review of the manuscript. Purified BLM was generously provided by Ian Hickson.

REFERENCES

1. Martin, G. M., Austad, S. N., and Johnson, T. E. (1996) Nat. Genet. 13, 25–34
2. Brosh, R. M., Jr., and Bohr, V. A. (2002) Exp. Gerontol. 37, 491–506
3. Poot, M., Gollahon, K. A., Emond, M. J., Silber, J. B., and Rajbohnitch, P. S. (2002) PASEB J. 16, 75–78
4. Martin, G. M., Sprague, C. A., and Epstein, C. J. (1970) Lab. Invest. 23, 86–92
5. Fukuchi, K., Martin, G. M., and Monnat, R. J., Jr. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5893–5897
6. Stefanini, M., Scappaticci, S., Lagonamarsi, P., Borroni, G., Berardesca, E., and Nuzzo, F. (1989) Mutation Res. 219, 179–185
7. Yannone, S. M., Roy, S., Chan, D. W., Murphy, M. B., Huang, S., Campisi, J., and Chen, D. J. (2001) J. Biol. Chem. 276, 38242–38248
8. Saintigny, Y., Makienko, K., Swanson, C., Emond, M. J., and Monnat, R. J., Jr. (2002) Mol. Cell. Biol. 22, 6971–6978
9. Shen, J., and Leeb, L. A. (2001) Mol. Cell. Biol. 185, 921–944
10. Imamura, O., Fujita, K., Itoh, C., Takeda, S., Furuichi, Y., and Matsumoto, T. (2001) Oncogene 21, 954–960
11. Gehkert, E., Bauers, B., Schinzel, M., Ruprecht, K. W., and Jonas, J. B. (1988) Human Genet. 80, 135–139
12. Ogburn, C. E., Oshima, J., Poot, M., Chen, R., Gollahon, K. A., Rajbohntich, P. S., and Martin, G. M. (1997) Human Genetics 101, 121–125
13. Weinfeld, M., Raoouli-Nia, A., Chaudhry, M. A., and Britten, R. A. (2001) Radiat. Res. 156, 584–589
14. Wallace, S. S. (1998) Radiat. Res. 150, 860–879
15. Wilson, D. M., and Thompson, L. H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12754–12757
16. Lindahl, T. (2000) Mutat. Res. 462, 129–135
17. Klungland, A., and Lindahl, T. (1997) EMBO J. 16, 3341–3348
18. Dantzer, F., Schreiber, V., Niedergang, C., Trucou, F., Flattet, E., De La R, G., Oliver, J., Roll, V., Menasie-de Murcia, J., and de Murcia, G. (1999) Biochimie (Paris) 81, 69–75
19. Zhou, J., Ahn, J., Wilson, S. H., and Prives, C. (2001) EMBO J. 20, 914–923
20. DeMott, M. S., Zigman, S., and Bambara, R. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4603–4608
21. Shimel, M., Spillare, E. A., Harris, C. C., and Leder, P. (1999) J. Biol. Chem. 274, 37795–37799
24. Brosh, R. M., Jr., Orren, D. K., Nehlin, J. O., Ravn, P. H., Kenny, M. K., Machwe, A., and Bohr, V. A. (1999) J. Biol. Chem. 274, 18341–18350
25. Shen, J. C., Gray, M. D., Oshima, J., and Loeb, L. A. (1996) Nucleic Acids Res. 26, 2879–2885
26. Brosh, R. M., Jr., von Kobbe, C., Sommers, J. A., Karmakar, P., Opresko, P. L., Pietrowski, J., Dianova, I., Dianov, G. L., and Bohr, V. A. (2001) EMBO J. 20, 5791–5801
27. Strauss, P. R., Beard, W. A., Patterson, T. A., and Wilson, S. H. (1997) J. Biol. Chem. 272, 18323–18330
28. Beard, W. A., and Wilson, S. H. (1995) Methods Enzymol. 256, 38–70
29. Slupphaug, G., Eftedal, I., Kavli, B., Bharati, S., Helle, N. M., Haug, T., Levine, D. W., and Krokan, H. E. (1995) Biochemistry 34, 128–138
30. Orren, D. K., Brosh Jr., R. M., Nehlin, J. O., Machwe, A., Gray, M. D., and Bohr, V. A. (1999) Nucleic Acids Res. 27, 3557–3566
31. Srivastava, D. K., Evans, R. K., Kumar, A., Beard, W. A., and Wilson, S. H. (1996) Biochemistry 35, 3728–3734
32. von Kobbe, C., Karmakar, P., Dawut, L., Opresko, P., Zeng, X., Brush, R. M., Jr., Hickson, I. D., and Bohr, V. A. (2002) J. Biol. Chem. 277, 22035–22044
33. Kedar, P. S., Kim, S. J., Robertson, A., Hou, E., Prasad, R., Horton, J. K., and Wilson, S. H. (2001) J. Biol. Chem. 276, 31111–31123
34. Prasad, R., Singshal, R. K., Srivastava, D. K., Molina, J. T., Tomkinson, A. E., and Wilson, S. H. (1996) J. Biol. Chem. 271, 16000–16007
35. Brosh, R. M., Jr., Li, J. L., Kenny, M. K., Karow, J. K., Cooper, M. P., Kureekattil, R. P., Hickson, I. D., and Bohr, V. A. (2000) J. Biol. Chem. 275, 23500–23508
36. Opresko, P. L., Laine, J. P., Brosh, R. M., Jr., Seidman, M. M., and Bohr, V. A. (2001) J. Biol. Chem. 276, 44677–44687
37. Prasad, R., Lavrik, O. I., Kim, S. J., Kedar, P., Yang, X. P., Vande Berg, B. J., and Wilson, S. H. (2001) J. Biol. Chem. 276, 32441–32444
38. Fygenson, D. K., and Goodman, M. F. (1987) J. Biol. Chem. 262, 27931–27935
39. Gray, M. D., Shen, J. C., Kamath-Loeb, A. S., Blank, A., Stepher, B. L., Martin, G. M., Oshima, J., and Loeb, L. A. (1997) Nat. Genet. 17, 100–103
40. Mohaghegh, P., Karow, J. K., Brosh, R. M., Jr., Bohr, V. A., and Hickson, I. D. (2001) Nucleic Acids Res. 29, 2843–2849
41. Brosh, R. M., Jr., Waheed, J., and Sommers, J. A. (2002) J. Biol. Chem. 277, 23236–23245
42. Wilson, S. H., and Kunkel, T. A. (2000) Nat. Struct. Biol. 7, 176–178
43. Bohr, V. A., Souza, P. N., Nyaga, S. G., Dianova, I., Dianov, G. L., Kraemer, K., Seidman, M. M., and Brosh, R. M., Jr. (2001) Environ. Mol. Mutagen. 38, 227–234
44. Machwe, A. Ganunis, R., Bohr, V. A., and Orren, D. K. (2000) Nucleic Acids Res. 28, 2762–2770
45. DeMott, M. S., Beyret, E., Wong, D., Bales, B. C., Hwang, J. T., Greenberg, M. M., and Demple, B. (2002) J. Biol. Chem. 277, 7637–7640
46. Lohman, T. M., and Bjornson, K. P. (1996) Annu. Rev. Biochem. 65, 169–214
47. Araujo, S. J., and Wood, R. D. (1999) Mutat. Res. 435, 23–33
48. Marti, T. M., Kunz, C., and Fleck, O. (2002) J. Cell. Physiol. 191, 28–41
49. Brosh, R. M., Jr., Majumdar, A., Desai, S., Hickson, I. D., Bohr, V. A., and Seidman, M. M. (2001) J. Biol. Chem. 276, 3024–3030
50. Constantinou, A., Tarsounas, M., Karow, J. K., Brosh, R. M., Bohr, V. A., Hickson, I. D., and West, S. C. (2000) EMBO Rep. 1, 80–84
51. Fry, M., and Loeb, L. A. (1999) J. Biol. Chem. 274, 12797–12802
52. Kamath-Loeb, A. S., Loeb, L. A., Johansson, E., Burgers, P. M., and Fry, M. (2001) J. Biol. Chem. 276, 16439–16446
53. Hartenstein, M. J., Goodman, M. F., and Petruska, J. (2002) J. Biol. Chem. 277, 41379–41389
54. Mian, I. S. (1997) Nucleic Acids Res. 25, 3187–3195
55. Kamath-Loeb, A. S., Shen, J. C., Loeb, L. A., and Fry, M. (1998) J. Biol. Chem. 273, 34145–34150
56. Huang, S., Beresten, S., Li, B., Oshima, J., Ellis, N. A., and Campisi, J. (2000) Nucleic Acids Res. 28, 2396–2405
57. Shevelev, I. V., and Hubscher, U. (2002) Nat. Rev. Mol. Cell. Biol. 3, 364–376
The Werner Syndrome Protein Stimulates DNA Polymerase β Strand Displacement Synthesis via Its Helicase Activity
Jeanine A. Harrigan, Patricia L. Opresko, Cayetano von Kobbe, Padmini S. Kedar, Rajendra Prasad, Samuel H. Wilson and Vilhelm A. Bohr

J. Biol. Chem. 2003, 278:22686-22695.
doi: 10.1074/jbc.M213103200 originally published online March 27, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M213103200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 57 references, 28 of which can be accessed free at http://www.jbc.org/content/278/25/22686.full.html#ref-list-1