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Enzymatic and DNA binding properties of purified WRN protein: high affinity binding to single-stranded DNA but not to DNA damage induced by 4NQO

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ABSTRACT

Mutations in the *WRN* gene result in Werner syndrome, an autosomal recessive disease in which many characteristics of aging are accelerated. A probable role in some aspect of DNA metabolism is suggested by the primary sequence of the *WRN* gene product. A recombinant His-tagged WRN protein (WRNp) was overproduced in insect cells using the baculovirus system and purified to near homogeneity by several chromatographic steps. This purification scheme removes both nuclease and topoisomerase contaminants that persist following a single Ni^{2+} affinity chromatography step and allows for unambiguous interpretation of WRNp enzymatic activities on DNA substrates. Purified WRNp has DNA-dependent ATPase and helicase activities consistent with its homology to the RecQ subfamily of proteins. The protein also binds with higher affinity to single-stranded DNA than to double-stranded DNA. However, WRNp has no higher affinity for various types of DNA damage, including adducts formed during 4NQO treatment, than for undamaged DNA. Our results confirm that WRNp has a role in DNA metabolism, although this role does not appear to be the specific recognition of damage in DNA.

INTRODUCTION

Werner syndrome (WS) is a rare autosomal recessive disease characterized by the early onset of many characteristics of human aging (reviewed in 1–5). The symptoms of the disease begin to appear after adolescence and include accelerated loss and graying of the hair, wrinkling and ulceration (particularly on the leg) of the skin, atherosclerosis, cataracts, osteoporosis and an increased risk of diabetes mellitus type II, hypertension and cancer. Individuals with WS almost always die before age 50, usually from myocardial infarction.

Studies using cultured cells from WS patients have revealed a number of abnormalities. Both primary and transformed WS cells are genomically more unstable than their normal counterparts, having elevated levels of chromosomal abnormalities, including insertions, deletions and translocations (6–13). Genomic instability in several human cancer-prone and premature aging syndromes has been shown to result from defects in repair of DNA damage (14,15). In agreement with this notion, some WS cells have subtle defects in removal of UV light-induced cyclobutane pyrimidine dimers from the transcribed strands of active genes (16) and telomeres (17). However, in comparison to normal cells, WS cells have no overt sensitivity to various DNA damaging agents (6,18,19), with the exception of 4-nitroquinoline-1-oxide (4NQO). When compared to their normal counterparts, WS cells treated with 4NQO have increased chromosomal abnormalities (13) and decreased cellular survival (20). Extracts from WS cells, although having normal levels of ligase activity, are prone to errors in ligation (21). Perhaps most importantly, WS primary fibroblasts lose their telomeric sequences at a faster rate than do normal fibroblasts (22,23). In accordance with this finding, primary fibroblasts from WS individuals have a reduced replicative capacity when compared to normal fibroblasts (7,18,19). It has been postulated that organismal aging is partially a consequence of loss of replicative capacity and cellular senescence (24), perhaps explaining the accelerated aging phenotype of WS.

The defective gene in WS, known as *WRN*, is located on chromosome 8 (25) and has recently been cloned and sequenced (26). The deduced 1432 amino acid sequence of WRN protein (WRNp) reveals several interesting features: (i) seven conserved motifs comprising a domain with homology to a family of DNA-dependent ATPases/helicases; (ii) a highly acidic domain; (iii) a nuclear localization signal; (iv) a tandemly repeated 27 amino acid sequence. In addition, the N-terminal part of WRNp has homology to the 3'→5' exonuclease domain of *Escherichia coli* DNA polymerase I (27,28). Homology in the central region of WRNp containing the seven helicase motifs places it in the RecQ subfamily of helicases that includes *E.coli* RecQ, *Saccharomyces cerevisiae* Sgs1, *Schizosaccharomyces pombe* rqh1p and human RecQL, RecQ4, RecQ5 and BLM proteins (1,26,29,30). Intriguingly,

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the BLM protein is defective in Bloom syndrome (29), a disease also characterized by high levels of genomic instability. Each mutation of *WRN* found in WS patients results in truncation of WRNp prior to the nuclear localization signal (31–33). This has led to speculation that such mutations will cause loss of function due to inability to locate to the nucleus and thus all result in the classic WS phenotype (34). Thus far, all evidence indicates that WRNp is a nuclear protein with a role in DNA metabolism.

In order to examine its potential roles in DNA metabolism, we have purified WRNp and characterized its properties. The *WRN* cDNA was attached to a hexahistidine tag and WRNp overproduced using a baculovirus/insect cell system. The recombinant WRNp was purified by several chromatographic steps and its properties were characterized. Our results indicate that WRNp is a DNA binding protein with higher affinity for single-stranded (ss)DNA than for double-stranded (ds)DNA. WRNp also does not have specific affinity for dsDNA containing single-strand breaks, photoproducts induced by UV irradiation, or 8-hydroxyguanine (8-OHdG) lesions. Importantly, WRNp does not bind with high affinity to DNA adducts induced by 4NQO, even though WS cells are hypersensitive to this carcinogenic agent. WRNp does have both DNA-stimulated ATPase activity and helicase activity, unwinding substrates containing at least 28 bp of dsDNA. In this report, we describe in detail the multistep purification of WRNp and the characterization of these activities.

MATERIALS AND METHODS

Materials

Spodoptera frugiperda cells (Sf9 strain) and *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA were obtained from Gibco BRL. *Escherichia coli* strain DR153 (*uvrB*$^-$) was obtained from the *E.coli* Genetic Stock Center (Yale University, New Haven, CT). *WRN* cDNA was a kind gift of J. Oshima and G. M. Martin (University of Washington, Seattle, WA). Oligonucleotides were purchased from Gibco BRL, Midland (dT_{16}) and Pharmacia [poly(dA) and poly(dT)]. Methylene blue and 4NQO were purchased from Ricca Chemical Co. and Sigma Chemical Co., respectively. DNase I was from Boehringer Mannheim. Human topoisomerase I was provided by A. Andersen (University of Aarhus, Aarhus, Denmark) and UvrA, UvrB and UvrC were gifts of L. Grossman (Johns Hopkins University, Baltimore, MD).

Construction of recombinant *WRN* bacmid DNA

Recombinant bacmid DNA was constructed using a commercially available kit (Invitrogen). Briefly, a *Sal*I–*Ssp*I fragment containing the full-length human *WRN* cDNA was subcloned into the *Sal*I and *Eco*RI cloning sites of pBlueBacHis2A, placing a hexahistidine tag at the N-terminus of the recombinant WRNp. A recombinant bacmid was generated and PCR analysis was performed to confirm the presence of *WRN* sequences. Recombinant bacmid DNA was purified by a miniprep procedure and then used for transfection into Sf9 insect cells as described below.

Production of recombinant virus and infection of insect cells

Sf9 cells were routinely maintained at a density of 0.5–3.0 × 10^6 cells/ml in suspension with aeration at 28°C in Grace's insect medium (Gibco BRL) supplemented with fetal bovine serum (10%), penicillin (10 U/ml) and streptomycin (10 μg/ml). Exponentially growing cells (9 × 10^5/well) were transferred into serum-free medium in 6-well dishes and then treated with mixtures of baculoviral DNA (recovered from *E.coli*) combined in various ratios with CellFECTIN reagent (Gibco BRL). After 6 h, the medium was removed and the cells were incubated in normal medium for 4–5 days. Intact recombinant baculovirus was recovered from the medium and kept at 4 or –80°C for short- and long-term storage, respectively. For further amplification of baculovirus, Sf9 cells were infected in T-100 or T-175 flasks and virus in the medium was harvested after at least 4 days at 28°C.

For optimal infection and overproduction of baculovirus-encoded recombinant WRNp, exponentially growing Sf9 cells (2 × 10^7/T175 flask) in monolayer were infected with baculovirus (multiplicity of infection = 10) and incubated at 28°C for 72–90 h. After this interval, both attached and suspended cells were combined, washed twice with phosphate-buffered saline (PBS) [plus 1.5 mM 4-(2-aminoethyl)benzene sulfonylfluoride (Calbiochem) in the second PBS wash] and collected by centrifugation. The cell pellets were stored at –80°C after removal of excess PBS. Overproduction of recombinant WRNp was confirmed by analysis of lysate from ~5 × 10^4 cells by SDS–PAGE (4%) followed by Coomassie staining.

Protein purification

Ni–NTA resin was from Qiagen; DEAE–Sepharose, Q Sepharose and SP Sepharose were from Pharmacia. DEAE–Sepharose and Q Sepharose chromatography was carried out using buffer A containing 150 mM Tris, pH 8.0, 10% glycerol, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitor mixture (2 μg/ml pepstatin, leupeptin, aprotinin and chymostatin) with the indicated concentrations of NaCl. Other purification buffers were as follows: (i) lysis buffer consisted of 150 mM Tris, pH 8.0, 10 mM (DEAE–Sepharose) or 150 mM (Ni–NTA) NaCl, 10% glycerol, 0.5% NP-40, 5 mM β-mercaptoethanol, 1 mM PMSF and protease inhibitor mixture; (ii) Ni wash buffer 1 consisted of 50 mM Tris, pH 8.0, 0.5 mM $LiCl_2$, 10% glycerol, 5 mM β-mercaptoethanol, 1 mM PMSF and protease inhibitor mixture; (iii) Ni wash buffer 2 consisted of 10 mM PIPES, pH 7.0, 50 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol, 1 mM PMSF and protease inhibitor mixture; (iv) Ni wash buffer 3 was the same as wash buffer 2 plus 10 mM imidazole; (v) Ni wash buffer 4 was the same as wash buffer 2 plus 25 mM imidazole; (vi) Ni elution buffer was 10 mM PIPES, pH 7.0, 50 mM NaCl, 300 mM imidazole, 20% glycerol, 5 mM β-mercaptoethanol and 1 mM PMSF; and (vii) SP buffer consisted of 150 mM Tris, pH 8.0, 20% glycerol, 5 mM β-mercaptoethanol and 1 mM PMSF supplemented with the indicated amounts of NaCl.

All steps of purification were carried out at 4°C. For purification by Ni–NTA chromatography only, Sf9 cells producing significant quantities of WRNp were suspended and lysed for 15 min in Ni–NTA lysis buffer, then insoluble material was pelleted by centrifugation (10 000 *g* for 10 min). This procedure succeeded in solubilizing 25–40% of WRNp produced in insect cells. Neither changing the infection incubation period nor using homogenization, other detergents and/or higher salt concentrations during lysis reproducibly improved solubilization of WRNp. The clarified lysate was bound in batch for 1 h to

Ni–NTA resin (pre-equilibrated in Ni–NTA lysis buffer) and a column was poured from the resin/lysate slurry. After unbound material was allowed to flow through, the column was sequentially washed with 20, 10, 10 and 20 column vol of Ni wash buffers 1–4, respectively, to remove non-specifically bound protein. WRNp was eluted from Ni–NTA resin using 10 column vol of Ni elution buffer. The solubility and stability of WRNp was maintained by immediate addition of bovine serum albumin (BSA) to a final concentration of 100 μg/ml. Mock protein purifications were prepared in an identical manner from cells infected with baculovirus containing no *WRN* sequences.

For purification by multiple chromatographic steps, clarified lysate was prepared as above except in DEAE lysis buffer, then loaded onto DEAE–Sepharose resin pre-equilibrated in DEAE lysis buffer. The resin was washed sequentially with buffer A containing 10 mM NaCl, then with buffer A containing 80 mM NaCl. WRNp was eluted from DEAE resin with buffer A containing 180 mM NaCl. Fractions containing the WRNp peak were pooled and the NaCl concentration was adjusted to 90 mM using buffer A minus NaCl. This preparation was then loaded onto a column of Q Sepharose resin equilibrated in buffer A containing 90 mM NaCl. The column was extensively washed with buffer A containing 100 mM NaCl, then with buffer A containing 200 mM NaCl. WRNp was eluted from Q Sepharose with buffer A containing 400 mM NaCl and fractions containing the WRNp peak were pooled. This material was loaded directly onto Ni–NTA resin equilibrated in buffer A containing 400 mM NaCl. As described above, Ni–NTA resin was washed with Ni wash buffers 1–4, WRNp was eluted with Ni elution buffer and BSA was added.

For some experiments, WRNp was further purified by immediately adding the Ni–NTA eluted material (including BSA) to SP Sepharose resin that had been pre-equilibrated in SP buffer plus 50 mM NaCl. After a 2 h batch binding step, the resin was sedimented by centrifugation (500 *g*, 5 min). The supernatant was removed and the resin was subsequently washed with 10 bed vol of the same buffer, then with 5 bed vol of SP buffer plus 100 mM NaCl. WRNp was eluted in 3–4 bed vol of SP buffer plus 400 mM NaCl. Protein fractions from Ni–NTA and SP Sepharose chromatography were immediately frozen and stored at –80ºC.

Nuclease and topoisomerase assays

For detection of nuclease (5'→3' exonuclease) activity in various WRNp preparations, a 5'-^{32}P-labeled 57 base oligomer was incubated with 25 or 50 ng of WRNp in buffer containing 100 mM Tris (pH 7.5), 10 mM $MgCl_2$, 500 μg/ml BSA and 10 mM dithiothreitol for 30 min at 25ºC. The DNA products were separated by non-denaturing polyacrylamide (6%) gel electrophoresis, then visualized by phosphorimaging (Molecular Dynamics). The appearance of mononucleotide products indicates the presence of a 5'→3' exonuclease in the preparation.

For detection of topoisomerase (type I) activities in various WRNp preparations, superhelical pKS plasmid DNA was incubated for 20 min at 37ºC with WRNp (0–300 ng) in buffer containing 10 mM Tris (pH 7.5), 100 mM NaCl and 0.5 mM EDTA. As a positive control, pKS was incubated with purified recombinant human topoisomerase I (50 ng) in the same buffer. Note that topoisomerase type II enzymes require ATP, but topoisomerase type I enzymes do not. The DNA products were electrophoresed on agarose (1%) gels and the DNA was visualized by ethidium bromide staining. The presence of topoisomerase type I activity was assessed by the conversion of superhelical plasmid molecules to more relaxed (slower migrating) DNA topoisomers.

DNA binding substrates

A 2973 base circular pKS (+) ssDNA and pKS dsDNA were obtained from Stratagene and double-stranded pKS was transfected into, amplified in and purified from *E.coli*. For creation of damaged DNA substrates *in vitro*, double-stranded pKS was linearized with *Ssp*I and either: (i) UV irradiated (1000 J/m^2 of 254 nm light); (ii) incubated with methylene blue (4 μM) and treated with fluorescent light for either 15 or 60 min; (iii) treated with DNase I (0.002 U/μg DNA) for 20 min. The extent of digestion of DNase I-treated plasmid was checked by comparison with the digestion of superhelical plasmid to the relaxed form on agarose gels and DNase I was removed using Micropure-EZ filters (Amicon). From the Poisson distribution, the approximate number of UV lesions was determined by measurement of the conversion of superhelical DNA molecules to the relaxed form after treatment with a combination of the UvrA, UvrB and UvrC proteins [Uvr(A)BC endonuclease] and fluoroimaging analysis of ethidium bromide-stained agarose (1%) gels.

Because intracellular enzymatic activation of 4-NQO is required for the formation of quinoline–DNA adducts (35,36), we devised a strategy to obtain plasmid DNA containing adducts induced by 4NQO treatment of bacterial cells (see Fig. 4). To minimize the repair of bulky adducts in plasmid DNA, we first transformed nucleotide excision repair-deficient *E.coli* strain DR153 (uvrB$^-$) with double-stranded pKS plasmid containing the ampicillin resistance gene. After selection and expansion of ampicillin-resistant colonies, cells from a 500 ml stationary phase culture of transformed strain DR153/pKS in LB were collected and washed in 500 ml of sodium phosphate buffer (67 mM, pH 6.8), then incubated at 37ºC for 1 h in 50 ml of phosphate buffer with 5 mM 4NQO. The drug was removed by sequential washing of the cells with phosphate buffer and the pKS plasmid was isolated and purified using a plasmid DNA purification kit (Qiagen). The presence and frequency of adducts formed by 4NQO treatment was assessed by the conversion of superhelical damaged DNA to the relaxed form after treatment of the plasmid with Uvr(A)BC endonuclease.

DNA binding assays

ssDNA and dsDNA (undamaged and damaged) substrates were incubated for 30 min at 25ºC with WRNp (2.4–216 nM) in buffer containing 100 mM Tris (pH 7.5), 10 mM $MgCl_2$, 200 mM NaCl, 500 μg/ml BSA and 10 mM dithiothreitol. DNA–protein mixtures (25 μl each) were analyzed by agarose (0.8 or 1.0%) gel electrophoresis at 4ºC. Gels were stained for 30 min in ethidium bromide or Syber Green I (Molecular Probes) and DNA was visualized using a Fluorimager SI (Molecular Dynamics). The amount of unretarded DNA was quantitated using ImageQuant software.

ATPase assay

The ATPase assay has been described in detail previously (37). The various DNA effectors used in ATPase reactions were pKS circular ssDNA, pKS (superhelical), pKS (relaxed), dT_{16}, poly(dT) and poly(dA). In controls for non-specific stimulatory

effects of polyanions, sodium acetate (50–500 mM) or sodium sulfite (50–500 mM) were substituted for DNA. ATPase reaction mixtures (30 µl) containing 40 mM Tris (pH 7.4), 4 mM $MgCl_2$, 5 mM dithiothreitol, 0.8 mM [^{3}H]ATP (42 c.p.m./pmol), recombinant WRNp (107 nM) and the indicated DNA effector (30 µM nucleotide) were incubated at 25ºC. Aliquots were removed at 2 min intervals and conversion of [^{3}H]ATP to [^{3}H]ADP was evaluated by thin layer chromatography and scintillation counting.

DNA helicase assay

The DNA substrate for helicase assays was constructed as previously described (38). Briefly, a 28 base oligomer complementary to positions 6296–6323 in M13mp18 (obtained from New England Biolabs) was 5'-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. After the radiolabeled 28mer was annealed to the M13mp18 ssDNA circle, the partially duplex DNA was purified by gel filtration chromatography using Bio-gel A-5M resin (Bio-Rad). The helicase reaction mixtures (20 µl) containing 40 mM Tris (pH 7.4), 4 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM ATP, 0.5 µg/ml yeast tRNA, recombinant WRNp (107 nM) and the 28 bp partial duplex substrate (2 µM nucleotide) were incubated at 25°C for the indicated times. The reactions were terminated by the addition of 10 µl of helicase loading dye (50 mM EDTA, 40% glycerol, 0.9% SDS, 0.1% bromophenol blue and 0.1% xylene cyanol) and the DNA products were resolved by electrophoresis on non-denaturing polyacrylamide (12%) gels. Radiolabeled DNA species were visualized by phosphorimaging (Molecular Dynamics) or autoradiography and the amount of unwinding was quantitated using ImageQuant software.

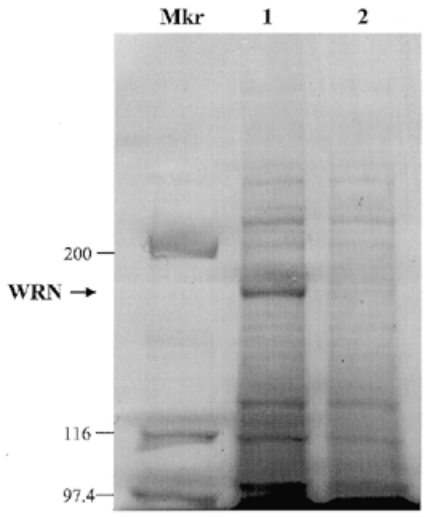

Figure 1. Overproduction of recombinant WRNp. Sf9 cells were incubated at 28ºC for 72 h with baculovirus containing either *WRN* cDNA (lane 1) or only viral DNA (lane 2) sequences. Approximately 5×10^4 cells from each infection were lysed in 25 µl of 1× SDS–PAGE loading buffer. The lysates were electrophoresed on an SDS–polyacrylamide (4%) gel along with protein molecular weight markers (Mkr) and the gel was stained with Coomassie brilliant blue. The size (in kDa) of the molecular weight markers and the position of the recomibinant WRNp band are denoted at the left. Note that maximal separation was achieved in the high molecular weight range by allowing cellular proteins of less than ~90 kDa to run off the gel.

RESULTS

Purification of WRNp

Insect cells infected with baculovirus carrying the *WRN* gene with an N-terminal His-tag were checked for production of WRNp by SDS–PAGE with Coomassie staining (Fig. 1). Under the infection conditions described in Materials and Methods, we obtained optimal overproduction of recombinant WRNp (~0.25 mg/10^7 cells) as measured in whole insect cell lysates (Fig. 1, lane 1), compared with a lysate from insect cells infected with baculovirus without *WRN* cDNA sequences (Fig. 1, lane 2).

Initially, only Ni–NTA affinity chromatography was used to partially purify recombinant WRNp containing the hexahistidine affinity tag. As judged by SDS–PAGE with Coomassie staining (Fig. 2A), WRNp purified by Ni–NTA chromatography was >60–80% pure, depending on variable overproduction and solubility of the recombinant protein in independent purifications. This protein preparation was suitable for use in ATPase and helicase assays, as mock-infected cell lysates purified by Ni–NTA chromatography contained no detectable ATPase or helicase activity (39). However, WRNp purified by only Ni–NTA chromatography does contain contaminating single-stranded nuclease (Fig. 2D) and topoisomerase I-type activities (Fig. 2E) that are also present in mock purifications (data not shown). Although the WRNp contains 3'→5' exonuclease activity (40,41), the nuclease contaminant was revealed to be a 5'→3' exonuclease by stepwise degradation of a 3'-^{32}P-labeled substrate (data not shown).

To remove these additional contaminants, recombinant WRNp was purified to near homogeneity using a series of chromatographic steps (described in Materials and Methods). A Coomassie-stained SDS–polyacrylamide gel of the WRN peak fractions from each chromatographic step in the extended purification is shown in Figure 2B. The protein (with BSA added for stabilization) is >75% following Ni–NTA chromatography (lane 4) and >90% following SP Sepharose chromatography (lane 5). Silver staining of the Ni–NTA and SP Sepharose peak fractions reveals the improvement in purity following SP Sepharose chromatography (Fig. 2C). Purified recombinant WRNp is stable for at least 6 months when stored at –80ºC.

Importantly, this extensive purification removes several contaminants that could potentially interfere with characterization of the biochemical activities of WRNp. As mentioned above, WRNp purified only by Ni–NTA affinity chromatography is contaminated with significant topoisomerase (type I) and 5'→3' exonuclease activities. However, when DEAE–Sepharose and Q Sepharose chromatography were performed prior to the Ni–NTA affinity chromatography, the resulting highly purified WRNp had little or no contaminating 5'→3' exonuclease activity on a ssDNA substrate when compared with Ni–NTA-purified WRNp (Fig. 2D). Although WRNp has itself been shown to have 3'→5' exonuclease activity (40,41), our results suggest that a ssDNA fragment is not an appropriate substrate for this activity. WRNp preparations, after additional chromatographic

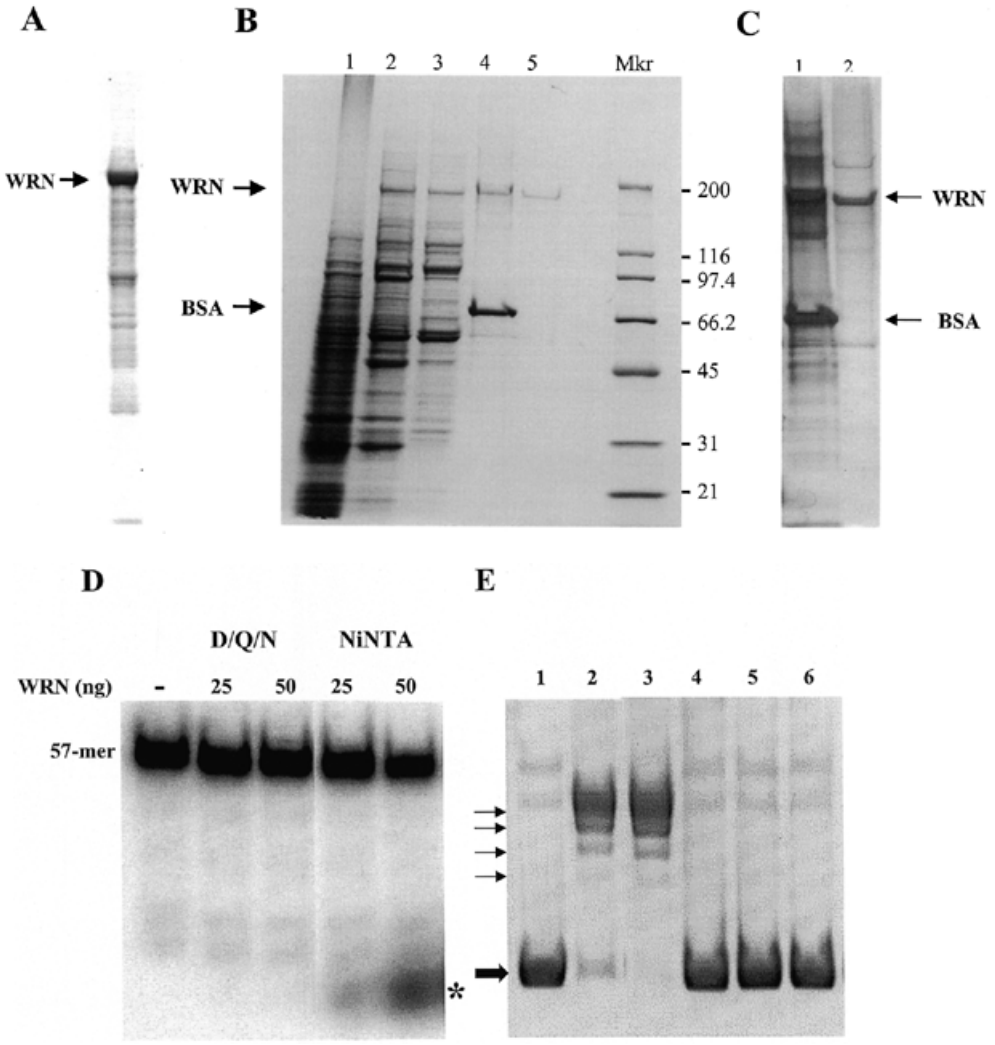

Figure 2. Purification of recombinant WRNp. (**A**) A Coomassie-stained SDS–PAGE (8–16% gradient gel) analysis of WRNp peak fraction purified from Sf9 cells by Ni–NTA affinity chromatography alone. (**B**) WRNp was purified using multiple column chromatography as demonstrated by Coomassie staining of an SDS–PAGE (8–16% gradient gel) analysis showing Sf9 cell lysate (lane 1) and WRNp peak fractions from DEAE–Sepharose (lane 2), Q Sepharose (lane 3), Ni–NTA (lane 4) and SP Sepharose (lane 5) chromatography as well as protein molecular weight markers (Mkr). The size (in kDa) and position of markers are denoted (at right) and the positions of WRNp and BSA (added to maintain stability) are denoted with arrows. (**C**) Silver staining analysis of Ni–NTA (lane 1) and SP Sepharose (lane 2) peak fractions from the same gel as in (B). The positions of WRN and BSA are denoted with arrows. (**D**) Assay for nuclease activity. A 5'-^{32}P-labeled 57mer was incubated at 25ºC with or without WRNp (25 or 50 ng) purified by Ni–NTA affinity chromatography alone (NiNTA, right) or by DEAE–Sepharose, Q Sepharose and Ni–NTA chromatography (D/Q/N, left). The products were analyzed on a non-denaturing polyacrylamide (6%) gel. The appearance of mononucleotide products (denoted by an asterisk) indicates the presence of contaminating 5'→3' exonuclease activity on ssDNA. (**E**) Assay for topoisomerase (type 1) activity. Superhelical pKS plasmid was incubated for 20 min at 37ºC with WRNp (200 ng) purified by Ni–NTA affinity chromatography (lane 3) or WRNp (100, 200 and 300 ng) purified by DEAE–Sepharose, Q Sepharose and Ni–NTA affinity chromatography (lanes 4–6, respectively). As controls, superhelical pKS was incubated without enzyme (lane 1) or with 50 ng of human topoisomerase I (lane 2). The DNA products were analyzed by agarose (1%) gel electrophoresis then visualized by ethidium bromide staining. The slower migrating, multiple bands (denoted by thin arrows) in the topoisomerase I control (lane 2) and in the Ni–NTA-purified WRNp reaction (lane 3) are more relaxed topoisomers of the plasmid pKS (original superhelical form denoted by thick arrow). The conversion of superhelical molecules to more relaxed topoisomers in the absence of ATP is indicative of topoisomerase (type I) activity.

steps, retained 3'→5' exonuclease activity but had reduced levels of 5'→3' exonuclease activity on dsDNA substrates, suggesting that the latter activity is not associated with WRNp (D.Orren, unpublished results). In addition, our multistep purification eliminated the contaminating topoisomerase (type I) activity (Fig. 2E). Obviously, the removal of these contaminating activities prevents misinterpretation of the endogenous WRNp activities on various types of DNA substrates.

ssDNA and dsDNA binding activities of WRNp

The amino acid sequence of WRNp contains putative exonuclease and helicase domains, indicating a possible role for

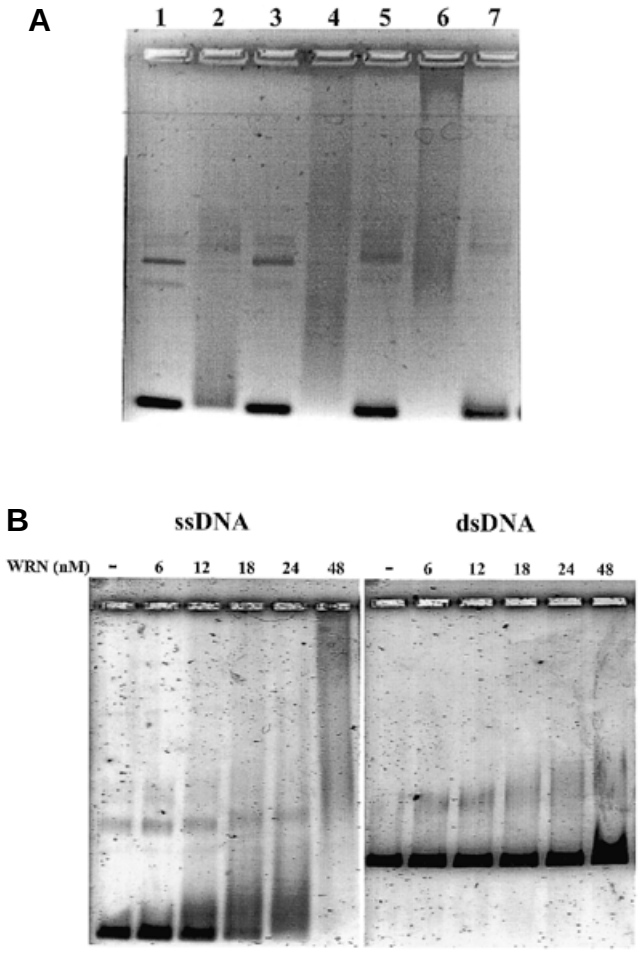

Figure 3. WRNp binding to ssDNA and dsDNA. (**A**) Single-stranded, circular pKS DNA (100 ng) was incubated alone (lane 1) or with equivalent volumes (1.5, 3 and 6 µl) of either WRNp (lanes 2, 4 and 6; 0.9, 1.8 and 3.6 pmol, respectively) or mock protein preparation (lanes 3, 5 and 7). The reactions were analyzed by agarose (1%) gel electrophoresis and the DNA was visualized by ethidium bromide staining. (**B**) WRNp (0, 6, 12, 18, 24 and 48 nM) was incubated for 30 min at 25ºC with 8 ng of either single-stranded (left) or double-stranded (right) pKS. DNA and DNA–protein mixtures were analyzed by agarose (0.8%) gel electrophoresis at 4°C and DNA was visualized by Syber Green staining and fluoroimaging. Retardation and smearing of DNA is evidence of the formation of DNA–protein complexes.

the protein in DNA metabolism. We first wanted to investigate the DNA binding capabilities of WRN protein. Binding of WRN to both ssDNA and dsDNA substrates was investigated by electrophoretic mobility shift analysis. As shown in Figure 3A, the mobility of a circular, ssDNA through an agarose gel is progressively slowed by the addition of increasing amounts of WRNp, while the addition of equivalent volumes of mock-purified preparations has little or no effect on the DNA substrate. Binding of WRNp to DNA substrates generates a broad distribution of species instead of a single shifted band, suggesting that multiple molecules of WRNp can be bound to a single DNA molecule, perhaps due to multiple binding sites for the protein and/or oligomerization properties of the protein itself. When circular, dsDNA was used as substrate, WRNp also demonstrated binding ability; again, mock-purified preparations showed little or no binding affinity (data not shown). A direct comparison of the ssDNA and dsDNA binding affinities over the same range of WRNp concentration shows that WRNp has a distinct preference for binding to ssDNA (Fig. 3B). The binding constant of WRNp for ssDNA, calculated from the WRNp concentration at which half of the DNA substrate is bound, is of the order of 10^8–10^9 M^{-1}, and at least 5-fold higher than for dsDNA.

DNA damaged by 4NQO treatment is not a high affinity substrate for WRNp

When compared with normal cells, cells from WS patients have elevated levels of genomic instability (6–13), suggesting that the normal function of WRNp might involve processing of some type of DNA damage. Moreover, the hypersensitivity and increased genomic instability of WS cells observed after treatment with the carcinogen 4NQO suggests that WRNp may play a direct role in the repair of 4NQO-induced DNA damage (13,20). A two-step intracellular enzymatic activation of 4NQO is required to generate a quinoline derivative that reacts with DNA to form several bulky guanine and adenine adducts (35,36). In addition, a minor amount of oxidative DNA damage is generated, including some 8-OHdG lesions (42) and strand breaks (43). To address the possibility that WRNp is involved in binding to 4NQO-induced DNA damage, a scheme was developed to obtain DNA containing quinoline adducts from cells treated with 4NQO (Fig. 4, top). Briefly, nucleotide excision repair deficient (*uvrB*$^-$) strain of *E.coli* that cannot repair bulky DNA lesions was transformed with the plasmid pKS and the resulting strain was incubated with 4NQO. Plasmid DNA was then purified and tested for the presence of lesions by incubation with Uvr(A)BC endonuclease, a nucleotide excision repair complex that incises DNA near the sites of bulky lesions, including guanine adducts formed by 4NQO (44,45). In these assays, addition of Uvr(A)BC endonuclease results in quantitative conversion of DNA from 4NQO-treated *E.coli* to the nicked form, while untreated DNA is not affected (Fig. 4, bottom left). This reflects the specific binding and incision by Uvr(A)BC endonuclease at sites of adducts created by 4NQO treatment. The average number of these 4NQO-induced [Uvr(A)BC endonuclease-sensitive] adducts is estimated by the Poisson distribution to be a minimum of four to five excinuclease-sensitive sites per plasmid. Higher levels of damage were unattainable due to the limited solubility of 4NQO in solvents compatible with the viability of *E.coli*. The actual number of 4NQO-induced adducts may be significantly higher than our measurements reveal, due to sub-optimal efficiency of endonuclease incision at damaged sites and the limitations of quantitation using the Poisson distribution.

Finally, we compared the binding affinity of WRNp to this plasmid containing 4NQO-induced lesions with its affinity for undamaged dsDNA, using gel retardation as described above. The experiment shown in Figure 4 (bottom right) demonstrates that neither DNA substrate was retarded at a low WRNp concentration (36 nM), while both the undamaged and the 4NQO-damaged substrate are shifted equally at a higher WRNp concentration (85 nM). Data from experiments measuring the binding affinity of WRNp (over a much wider range of

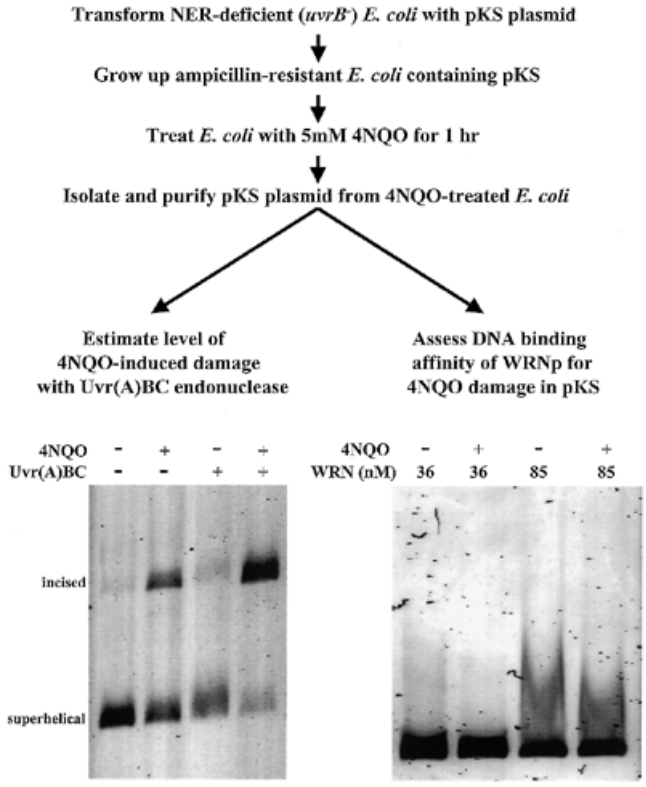

Figure 4. Binding of WRNp to DNA substrate isolated from 4NQO-treated cells. (Top) The protocol for isolating DNA containing damage induced by 4NQO. (Lower left) Uvr(A)BC endonuclease incision of 4NQO-damaged plasmid. pKS plasmid (200 ng) purified from either 4NQO-treated (+) or untreated (–) *E.coli* was incubated at 37ºC with Uvr(A)BC endonuclease [UvrA (20 nM), UvrB (375 nM) and UvrC (100 nM)]. The DNA products were then separated by agarose (1%) gel electrophoresis and visualized by ethidium bromide staining and fluoroimaging. (Lower right) WRNp binding to undamaged and 4NQO-treated pKS plasmid DNA from either 4NQO-treated (+) or untreated (–) *E.coli* was incubated with WRNp (36 or 85 nM) as indicated. The samples were analyzed by agarose (1%) gel electrophoresis at 4°C and DNA was visualized with Syber Green staining and fluoroimaging.

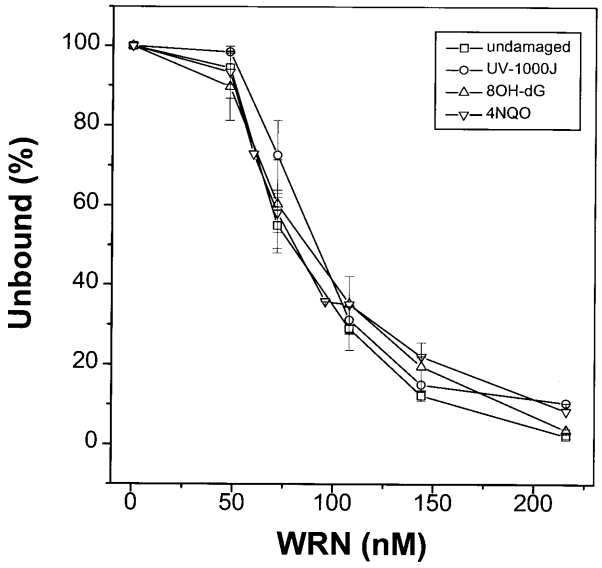

Figure 5. Comparison of WRNp binding activity on undamaged and damaged DNA. Undamaged DNA and DNA damaged with UV (1000 J/m^2) or methylene blue plus light (60 min) were prepared as described in Materials and Methods. The other damaged substrate was isolated from *E.coli* treated with 5 mM 4NQO as described in Materials and Methods and Figure 4. Each DNA substrate (10 ng) was incubated with increasing concentrations (0–270 nM) of WRNp and DNA–protein complexes were analyzed as described in Figure 3. The amounts of unretarded DNA were quantitated by fluoroimaging using ImageQuant software, normalized to the amount of unretarded DNA in the absence of WRNp and plotted versus WRNp concentration for each substrate. DNA substrates are indicated as untreated (undamaged, square) or treated with UV (UV-1000J, circle), methylene blue plus light (8-OHdG, triangle), or *in vivo* with 4NQO (4NQO, downward triangle). Each point is the average of two or three independent experiments.

concentration) are included in Figure 5. Again, WRNp binds with relatively equal affinity to undamaged DNA and DNA containing lesions generated by 4NQO. These results indicate that DNA lesions formed after 4NQO treatment are not high affinity binding sites for WRNp.

We also tested the ability of WRNp to bind to dsDNA containing various other types of DNA damage, again using gel mobility shift assays with undamaged DNA as a control. Binding to a fixed amount of each DNA substrate was measured over a range of WRNp concentration and the amount of unbound DNA remaining at each concentration was determined. WRNp does bind to both undamaged and damaged dsDNA substrates. However, DNA treated with UV light (containing >10 photoproducts/plasmid) was no better substrate for WRNp than undamaged DNA (Fig. 5). This suggests that the two major DNA adducts created by UV irradiation, cyclobutane pyrimidine dimers and 6–4 photoproducts, are not high affinity binding sites for WRNp. The same conclusion can also be made for DNA damaged with methylene blue plus light containing betweeen 5 and 10 8-OHdG adducts/plasmid (M.Anson, unpublished results), as well as other minor oxidative adducts (Fig. 5). WRNp also appears not to have a specific affinity for DNA containing single-strand breaks created by DNase I treatment (data not shown). These results demonstrate that several types of DNA damage are not specifically recognized by WRNp.

WRN ATPase activity

The amino acid sequence of WRNp contains motifs that place it in a large family of proteins with nucleic acid-dependent ATPase and helicase activities. We examined the ability of WRNp to hydrolyze ATP in the presence of various nucleic acid effectors (Table 1). Hydrolysis of ATP by WRNp alone was undetectable under the conditions of our assay. However, when different DNA effectors were added, each was able to stimulate WRNp ATPase activity. We also tested the effect of other chaotropic polyanions on ATP hydrolysis by WRNp. In the absence of DNA cofactors, acetate or sulfite (at concentrations up to 500 mM) did not significantly stimulate ATP hydrolysis. These results indicate that the DNA-dependence of ATP hydrolysis by WRNp is specific and cannot be mimicked by

other polyanionic species. Importantly, we were not able to detect any ATP hydrolysis by either a mock-purified preparation or the WRN-K577M protein with a mutation at an absolutely conserved Lys in helicase motif I (data not shown). Thus, ATP hydrolysis is an intrinsic activity of the wild-type WRNp and intact ATPase/helicase motifs are required for this activity.

Table 1. Hydrolysis of ATP (k_{cat}) catalyzed by WRNp[a] in the presence of various DNA effectors

DNA effector	k_{cat} (min^{-1})
None	ND[b]
dT_{16}	53.1 ± 6.3
poly (dT)[c]	150 ± 19
poly (dA)[d]	138 ± 10
pKS ss[e] (circle)	219 ± 14
pKS ds[e] (superhelical circle)[f]	36.5 ± 12.0
pKS ds[e] (relaxed circle)[f,g]	9.8 ± 2.9

[a]WRNp concentration in the ATP reactions was 107 nM.
[b]ND, not detectable.
[c]The approximate length of poly(dT) was 263 nt.
[d]The approximate length of poly(dA) was 401 nt.
[e]ss, single-stranded; ds, double-stranded.
[f]Form I (superhelical, closed circular) of plasmid pKS (2973 bp) was purified by CsCl and sucrose gradient centrifugation prior to use as an effector.
[g]Purified form I pKS was treated with topoisomerase II under conditions that resulted in >95% of DNA as form II (relaxed, closed circular plasmid).

Table 1 reveals several properties of the WRNp ATPase. The ATPase activity of WRNp is stimulated to a much greater degree by single-strand effectors [dT_{16}, poly(dT) and poly(dA)] than by double-stranded effectors (pKS, superhelical and relaxed). dsDNA effectors are ~5- to 10-fold less effective than long ssDNA effectors in stimulating WRNp ATPase activity. Moreover, WRNp-catalyzed ATP hydrolysis in the presence of covalently closed supercoiled pKS DNA is ~2-fold greater than in the presence of relaxed pKS. This suggests that superhelicity can induce some ssDNA character within the plasmid, in turn leading to greater stimulation of ATPase than with the relaxed plasmid molecule. This explanation is consistent with our observation that WRNp ATPase activity is greater in the presence of single-stranded effectors than double-stranded effectors. These results on the ATPase activity of WRNp are also in agreement with the higher affinity of WRNp for ssDNA than for dsDNA.

There are also differences in the ability of the individual ssDNA effectors to stimulate ATP hydrolysis by WRNp (Table 1). Although both polypurine ([poly(dA)] and polypyrimidine [poly(dT)] tracts appear to serve equally well as DNA effectors, the longer ssDNA molecules [single-stranded pKS and poly(dT) and poly(dA)] were significantly more effective in stimulating ATP hydrolysis by WRNp than the short (16 nt) oligomer (dT_{16}). This indicates that the ability to translocate along long stretches of ssDNA without additional binding steps is correlated with maximal ATP hydrolysis and suggests that WRNp translocates along ssDNA processively. Other helicases, such as *E.coli* UvrD, show similar behavior (46).

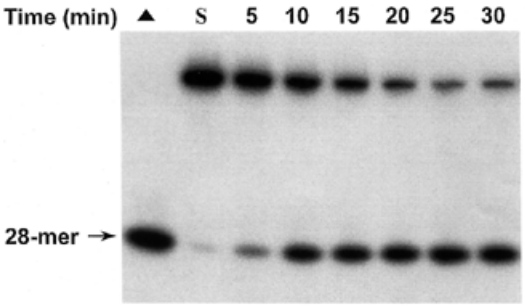

Figure 6. Helicase activity of WRNp on 28 bp duplex DNA substrate. Helicase assays were carried out as described in Materials and Methods. WRNp (107 nM) was incubated at 25ºC for 5–30 min with the ^{32}P-end-labeled 28 bp duplex DNA substrate. The DNA products were separated by non-denaturing polyacrylamide gel electrophoresis and visualized by phosphorimaging. As controls, the heat-denatured (filled triangle) and non-denatured (S) DNA substrate without WRNp were also analyzed. An arrow denotes the position of the displaced 28mer.

WRN helicase activity

Next, we tested the WRNp for DNA helicase activity by measuring its ability to displace a 28 base oligonucleotide annealed to a ssDNA circle. A kinetic analysis of helicase activity using Ni–NTA-purified recombinant WRN protein (Fig. 6) shows that detectable unwinding of this substrate occurs within 5 min and continues in a linear fashion over a 30 min interval. In contrast, a preparation of recombinant WRNp with a mutation (K577M) in one of the conserved helicase motifs does not detectably unwind this substrate (39). Thus, helicase activity is a *bona fide* property of wild-type WRNp. Quantitation of the data indicates that the initial rate of the reaction is ~2% unwound/min/pmol protein. These results confirm and extend earlier findings on the 3'→5' helicase activity associated with WRNp (47–49).

DISCUSSION

Patients with WS have *WRN* gene mutations that cause loss of function of WRNp, eventually resulting in the characteristic premature aging phenotype. The primary sequence of WRNp suggests a role in DNA metabolism that would be consistent with the genomic instability and premature replicative senescence properties of cells from WS patients. To obtain WRNp for detailed functional studies, we overexpressed a recombinant His-tagged human WRNp in insect cells infected with baculovirus containing the full-length *WRN* cDNA sequence, then purified the recombinant protein by chromatographic methods. Using only Ni^{2+} affinity chromatography designed for His-tagged proteins, WRNp can be purified to a high degree, but contaminants having exonuclease and topoisomerase (type I) activities co-elute with WRNp. Nuclease contaminants in WRNp preparations purified only by Ni^{2+} chromatography have been observed previously (40), but topoisomerase contaminants

have not been reported. Obviously, the presence of such contaminants could result in ambiguity in or misinterpretation of the activities of WRNp on DNA substrates. To obtain WRNp preparations suitable for use in such assays, we developed a procedure using additional chromatographic steps to remove these exonuclease and topoisomerase contaminants. Thus, our multistep purification of WRNp is a significant improvement over earlier methods (40,47,49) and yields WRNp that is much more useful in characterization of its enzymatic activities on DNA substrates.

The primary amino acid sequence of WRNp contains a central domain comprised of seven motifs that places it in a large family of proteins with DNA-dependent ATPase and/or helicase activity. We assayed purified WRNp for both hydrolysis of ATP and unwinding of DNA substrates. Our results demonstrate that ATP hydrolysis by WRNp is DNA dependent. ATP hydrolysis is stimulated to a much greater degree by ssDNA than by dsDNA effectors. Longer ssDNA substrates (>250 nt) are more effective at stimulating ATP hydrolysis by WRNp than short ssDNA substrates (16 nt), presumably due to the intimate connection between ATP hydrolysis and translocation along ssDNA. Previous reports have also detected nucleotide hydrolysis by WRNp (48,49). However, our maximum k_{cat} of ~200 min^{-1} is more in line with the values generally reported for helicases (ranging from 10^3 to 10^4) (50,51) than an earlier reported value of ~10^6 min^{-1} for the k_{cat} of ATP hydrolysis by WRNp (49). Importantly, WRNp containing a mutation in helicase motif I that changes an absolutely conserved Lys residue to Met does not detectably hydrolyze ATP (39,47). Our results indicate that WRNp, in a reaction dependent on Mg^{2+} and ATP, has helicase activity on a 28 bp duplex substrate. Neither the mutant WRNp described above nor a mock-purified preparation detectably unwinds this substrate. The finding that mutant WRNp and mock-purified preparations do not catalyze either ATP hydrolysis or DNA unwinding indicates that these activities are attributable to wild-type WRNp. Notably, mutation of the conserved Lys residue in other helicase family members have previously been demonstrated to eliminate both ATPase and helicase activity (52–56). Our results confirm earlier reports that demonstrated WRNp-catalyzed unwinding of short duplex regions (47,49).

The DNA-dependent ATPase and DNA unwinding properties of WRNp point to a definite role for WRNp in DNA metabolism. To address this role, we examined the DNA binding properties of WRNp. Our results indicate that although WRNp binds to both ssDNA and dsDNA, its affinity is at least 5-fold higher for ssDNA. The binding constant for WRNp to ssDNA is ~10^8–10^9 M^{-1}. The preferential binding of WRNp to ssDNA is typical of proteins with helicase activity (50,57). The preferential binding and ATP hydrolysis of WRNp in the presence of ssDNA is consistent with a role in processes (such as repair, recombination or replication) that generate ssDNA intermediates.

The genomic instability phenotype of WRN cells suggests that a DNA repair deficiency could be the primary biochemical defect. Moreover, WRN cells are hypersensitive to 4NQO (13,20), an agent that induces formation of several bulky DNA adducts. Adducts formed by 4NQO are thought to be removed by nucleotide excision repair, a general DNA repair pathway that acts on a wide variety of DNA damage (reviewed in 58–60). Thus, WRNp could potentially be involved in recognition and/or repair of certain DNA adducts. We tested the ability of WRNp to bind to DNA substrates treated with different types of damaging agents, including 4NQO. In comparison to binding of undamaged dsDNA, our results indicate that WRNp does not bind with a significantly higher affinity to dsDNA treated with UV irradiation, methylene blue plus light or DNase I, containing UV photoproducts, 8-OHdG adducts and other oxidative lesions or single-stranded nicks, respectively. Notably, a DNA substrate containing adducts formed after 4NQO treatment was also not a high affinity substrate for WRNp. These results suggest that WRNp may not be involved in direct binding to bulky lesions or in a damage recognition step of a DNA repair pathway, although we cannot rule out the possibility that the histidine tag on the recombinant WRNp affects its DNA binding properties. Recently, a shift in the subcellular localization of WRNp from the nucleolus to the nucleus was observed after treatment of cells with 4NQO, UV or H_2O_2 (61). Our results suggest that this redistribution of WRNp is not due to migration and binding of WRNp to damaged nucleotides in DNA. Although we certainly cannot rule out a role for WRNp in DNA repair, our results are consistent with some studies that have shown normal cellular sensitivity and repair activity after treatment of WRN cells with UV and ionizing radiation (6,13,18). However, WRNp could be involved in binding to DNA damage not within the scope of this study or in post-recognition steps of a DNA repair pathway.

In summary, we have purified WRNp to near homogeneity and examined it for various enzymatic activities. WRNp is a DNA-dependent ATPase and helicase with higher affinity for ssDNA than for dsDNA. Moreover, several types of damaged DNA are not high affinity substrates for WRNp. WRNp has also recently been shown to be a 3'→5' exonuclease (40,41,62), as predicted by sequence homology in the N-terminal region of the protein (27,28). These results confirm that WRNp has an as yet undefined role in DNA metabolism. Characterization of the enzymatic activities and binding properties of WRNp on more complex DNA substrates should be helpful in determining the precise function of WRNp.

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REFERENCES

1. Lombard,D.B. and Guarente,L. (1996) *Trends Genet.*, **12**, 283–286.
2. Goto,M. (1997) *Mech. Ageing Dev.*, **98**, 239–254.
3. Brown,W.T. (1990) *Annu. Rev. Gerontol. Geriatr.*, **10**, 23–42.
4. Dyer,C.A.E. and Sinclair,A.J. (1998) *Age Ageing*, **27**, 73–80.
5. Martin,G.M. (1997) *Phil. Trans. R. Soc. Lond.*, **352**, 1773–1780.
6. Stefanini,M., Scappaticci,S., Lagomarsini,P., Borroni,G., Berardesca,E. and Nuzzo,F. (1989) *Mutat. Res.*, **219**, 179–185.
7. Melaragno,M.I., Pagni,D. and de Arruda Cardoso Smith,M. (1995) *Mech. Ageing Dev.*, **78**, 117–122.
8. Fukuchi,K.-I., Martin,G.M. and Monnat,R.J.,Jr (1989) *Proc. Natl Acad. Sci. USA*, **86**, 5893–5897.
9. Nordenson,I. (1997) *Hereditas*, **87**, 151–154.
10. Salk,D., Au,K., Hoehn,H. and Martin,G.M. (1981) *Cytogenet. Cell Genet.*, **30**, 92–107.
11. Fukuchi,K.-I., Tanaka,K., Kumahara,Y., Marumo,K., Pride,M.B., Martin,G.M. and Monnat,R.J.,Jr (1990) *Hum. Genet.*, **84**, 249–252.

12. Weirich-Schwaiger,H., Weirich,H.G., Gruber,B., Schweiger,M. and Hirsch-Kauffmann,M. (1994) *Mutat. Res.*, **316**, 37–48.
13. Gebhardt,E., Bauer,R., Raub,U., Schinzel,M., Ruprecht,K.W. and Jonas,J.B. (1988) *Hum. Genet.*, **80**, 135–139.
14. Friedberg,E.C., Walker,G.C. and Siede,W. (1995) *DNA Repair and Mutagenesis*. ASM Press, Washington, DC, pp. 633–685.
15. Bohr,V.A., Dianov,G.D., Balajee,A.S., May,A. and Orren,D.K. (1998) *J. Invest. Dermatol. Symp. Proc.*, **3**, 11–13.
16. Webb,D.K., Evans,M.K. and Bohr,V.A. (1996) *Exp. Cell Res.*, **224**, 272–278.
17. Kruk,P.A., Rampino,N.J. and Bohr,V.A. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 258–262.
18. Fujiwara,Y., Higashikawa,T. and Tatsumi,M. (1977) *J. Cell Physiol.*, **92**, 365–374.
19. Saito,H. and Moses,R.E. (1991) *Exp. Cell Res.*, **192**, 373–379.
20. Ogburn,C.E., Oshima,J., Poot,M., Chen,R., Hunt,K.E., Gollahon,K.A., Rabinovitch,P.S. and Martin,G.M. (1997) *Hum. Genet.*, **101**, 121–125.
21. Runger,T.M., Bauer,C., Dekant,B., Moller,K., Sobotta,P., Czerny,C., Poot,M. and Martin,G.M. (1994) *J. Invest. Dermatol.*, **102**, 45–48.
22. Tahara,H., Tokutake,Y., Maeda,S., Kataoka,H., Watanabe,T., Satoh,M., Matsumoto,T., Sugawara,M., Ide,T., Goto,M., Furuichi,Y. and Sugimoto,M. (1997) *Oncogene*, 1911–1920.
23. Schulz,V.P., Zakian,V.A., Ogburn,C.E., McKay,J., Jarzebowicz,A., Edland,S.D. and Martin,G.M. (1996) *Hum. Genet.*, **97**, 750–754.
24. Campisi,J. (1996) *Cell*, **84**, 497–500.
25. Goto,M., Rubenstein,M., Weber,J., Woods,K. and Drayna,D. (1992) *Nature*, **355**, 735–738.
26. Yu,C.-E., Oshima,J., Fu,Y.-H., Wijsman,E.M., Hisama,F., Alisch,R., Matthews,S., Nakura,J., Miki,T., Ouais,S., Martin,G.M., Mulligan,J. and Schellenberg,G.D. (1996) *Science*, **272**, 258–262.
27. Mushegian,A.R., Bassett,D.E.,Jr, Boguski,M.S., Bork,P. and Koonin,E.V. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 5831–5836.
28. Moser,M.J., Holley,W.R., Chatterjee,A. and Mian,I.S. (1997) *Nucleic Acids Res.*, **25**, 5110–5118.
29. Ellis,N.A., Groden,J., Ye,T.-Z., Straughen,J., Lennon,D.J., Ciocci,S., Proytcheva,M. and German,J. (1995) *Cell*, **83**, 655–666.
30. Kitao,S., Ohsugi,I., Ichikawa,K., Goto,M., Furuichi,Y. and Shimamoto,A. (1998) *Genomics*, **54**, 443–452.
31. Goto,M., Imamura,O., Kuromitsu,J., Matsumoto,T., Yamabe,Y., Tokutake,Y., Suzuki,N., Mason,B., Drayna,D., Sugawara,M., Sugimoto,M. and Furuichi,Y. (1997) *Hum. Genet.*, **99**, 191–193.
32. Oshima,J., Yu,C.-E., Piussan,C., Klein,G., Jabkowski,J., Balci,S., Miki,T., Nakura,J., Ogihara,T., Ells,J., Smith,M., Melaragno,M.I., Fraccaro,M., Scappaticci,S., Matthews,J., Ouais,S., Jarzebowicz,A., Schellenberg,G.D. and Martin,G.M. (1996) *Hum. Mol. Genet.*, **5**, 1909–1913.
33. Yu,C.-E., Oshima,J., Wijsman,E.M., Nakura,J., Miki,T., Piussan,C., Matthews,S., Fu,Y.-H., Mulligan,J., Martin,G.M., Schellenberg,G.D. and Werner's Syndrome Collaborative Group (1997) *Am. J. Hum. Genet.*, **60**, 330–341.
34. Matsumoto,T., Shimamoto,A., Goto,M. and Furuichi,Y. (1997) *Nature Genet.*, **16**, 335–336.
35. Bailleul,B., Gallegue,S. and Loucheux-Lefebvre,M.-H. (1981) *Cancer Res.*, **41**, 4559–4565.
36. Gallegue-Zouitina,S., Bailleul,B. and Loucheux-Lefebvre,M.-H. (1985) *Cancer Res.*, **45**, 520–525.
37. Scott,J.F., Eisenberg,S., Bertsch,L.L. and Kornberg,A. (1977) *Proc. Natl Acad. Sci. USA*, **74**, 193–197.
38. Matson,S.W. (1986) *J. Biol. Chem.*, **261**, 10169–10175.
39. Brosh,R.M., 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.
40. Huang,S., Li,B., Gray,M.D., Oshima,J., Mian,I.S. and Campisi,J. (1998) *Nature Genet.*, **20**, 114–116.
41. Shen,J.-C., Gray,M.D., Oshima,J., Kamath-Loeb,A.S., Fry,M. and Loeb,L.A. (1998) *J. Biol. Chem.*, **273**, 34139–34144.
42. Kohda,K., Tada,M., Kasai,H., Nishimura,S. and Kawazoe,Y. (1986) *Biochem. Biophys. Res. Commun.*, **139**, 626–632.
43. Menichini,P., Fronza,G., Tornaletti,S., Gallegue-Zouitina,S., Bailleul,B., Loucheux-Lefebvre,M.-H., Abbondandolo,A. and Pedrini,A.M. (1989) *Carcinogenesis*, **10**, 1589–1593.
44. Snyderwine,E.G. and Bohr,V.A. (1992) *Cancer Res.*, **52**, 4183–4189.
45. Thomas,D.C., Husain,I., Chaney,S.G., Panigrahi,G.B. and Walker,I.G. (1991) *Nucleic Acids Res.*, **19**, 365–370.
46. Matson,S.W. and George,J.W. (1987) *J. Biol. Chem.*, **262**, 2066–2076.
47. Gray,M.D., Shen,J.-C., Kamath-Loeb,A.S., Blank,A., Sopher,B.L., Martin,G.M., Oshima,J. and Loeb,L.A. (1997) *Nature Genet.*, **17**, 100–103.
48. Shen,J.-C., Gray,M.D., Oshima,J. and Loeb,L.A. (1998) *Nucleic Acids Res.*, **26**, 2879–2885.
49. Suzuki,N., Shimamoto,A., Imamura,O., Kuromitsu,J., Kitao,S., Goto,M. and Furuichi,Y. (1997) *Nucleic Acids Res.*, **25**, 2973–2978.
50. Lohman,T.M. and Bjornson,K.P. (1996) *Annu. Rev. Biochem.*, **65**, 169–214.
51. Brosh,R.M. and Matson,S.W. (1997) *J. Biol. Chem.*, **272**, 572–579.
52. Walker,J.M., Sarsate,M., Runswick,M.J. and Gay,N.J. (1982) *EMBO J.*, **1**, 945–951.
53. George,J.W., Brosh,R.M. and Matson,S.W. (1994) *J. Mol. Biol.*, **235**, 424–435.
54. Sung,P., Higgins,D., Prakash,L. and Prakash,S. (1988) *EMBO J.*, **7**, 3263–3269.
55. Notarnicola,S.M. and Richardson,C.C. (1993) *J. Biol. Chem.*, **268**, 27198–27207.
56. Patel,S.S., Hingorani,M.M. and Ng,W.M. (1994) *Biochemistry*, **33**, 7857–7868.
57. Matson,S.W. and Kaiser-Rogers,K.A. (1990) *Annu. Rev. Biochem.*, **59**, 289–329.
58. van Houten,B. (1990) *Microbiol. Rev.*, **54**, 18–51.
59. Sancar,A. (1996) *Annu. Rev. Biochem.*, **65**, 43–81.
60. Wood,R.D. (1996) *Annu. Rev. Biochem.*, **65**, 135–167.
61. Gray,M.D., Wang,L., Youssoufian,H., Martin,G.M. and Oshima,J. (1998) *Exp. Cell Res.*, **242**, 487–494.
62. Kamath-Loeb,A.S., Shen,J.-C., Loeb,L.A. and Fry,M. (1998) *J. Biol. Chem.*, **273**, 34145–34150.