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Werner Syndrome Protein

I. DNA HELICASE AND DNA EXONUCLEASE RESIDE ON THE SAME POLYPEPTIDE*

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From the ‡Gottstein Memorial Cancer Research Laboratory, Departments of Pathology and Biochemistry, University of Washington, Seattle, Washington 98195-7705 and the §Unit of Biochemistry, Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, P. O. Box 9649, Haifa 31096, Israel

Werner Syndrome (WS) is a human progeroid disorder characterized by genomic instability. The gene defective in WS encodes a 3′ → 5′ DNA helicase (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) *Nat. Genet.* 17, 100–103). Sequence alignment analysis identified an N-terminal motif in WRN that is homologous to several exonucleases. Using combined molecular genetic, biochemical, and immunochemical approaches, we demonstrate that WRN also exhibits an integral DNA exonuclease activity. First, whereas wild-type recombinant WRN possesses both helicase and exonuclease activities, mutant WRN lacking the nuclease domain does not display exonucleolytic activity. In contrast, WRN proteins with defective helicase activity are active in exonucleolytic digestion of DNA. Second, the exonuclease co-purifies with the 160-kDa WRN protein and its associated DNA helicase and ATPase activities through successive steps of ion exchange and affinity chromatography, suggesting that all three activities are physically associated. Lastly, anti-WRN antiserum specifically coprecipitates the WRN helicase and exonuclease activities indicating that both activities reside on the same antigenic WRN polypeptide. The association of an exonuclease with WRN distinguishes it from other RecQ homologs and raises the possibility that the distinct phenotypic characteristics of WS may be due in part to a defective exonuclease.

Werner Syndrome (WS)[1] is an inherited disease characterized by an early onset of atherosclerosis, osteoporosis, diabetes mellitus, and cancers of non-epithelial cell origin (1, 2). Cultured cells from WS patients also present a shortened replicative lifespan (3) and increased genetic instability (4, 5). The genomic instability of WS cells is manifested at the cytogenetic level in the form of chromosome breaks and translocations, and at the molecular level predominately by multiple, large DNA deletions (4, 5).

The gene responsible for the WS phenotype has been identified, cloned, and sequenced (6). The cDNA encodes a protein (hereafter designated as WRN) of 1432 amino acids with a central helicase domain that is homologous in sequence to members of the RecQ family of DNA helicases. This family includes *Escherichia coli* RecQ, *Saccharomyces cerevisiae* Sgs-1p, *Schizosaccharomyces pombe* Rqh-1p, human RecQL, and the protein associated with Bloom Syndrome, BLM. All of these proteins share the seven sequence motifs common to helicases, including the characteristic motif II DExH box (7). The prototype of this family, the bacterial RecQ protein, is an active helicase that unwinds DNA in the 3′ → 5′ direction (8). Likewise, as predicted from DNA sequence alignments, the yeast Sgs-1p and the human RecQL, WRN, and BLM proteins have recently been demonstrated to exhibit ATP-dependent, 3′ → 5′ DNA unwinding activities *in vitro* (9–13).

The RecQ family of DNA helicases is believed to participate in numerous DNA transactions such as replication, recombination, and repair. *E. coli* RecQ is believed to initiate homologous recombination and suppress illegitimate recombination and the generation of aberrant recombination products (14, 15). Further, RecQ is proposed to play a role in the reassembly of replication forks disrupted by UV lesions (16, 17). The phenotypes of mutations in the eukaryotic homologs also suggest their involvement in these processes. *S. cerevisiae SGS-1* mutants display increased frequency of chromosome missegregation and recombination within ribosomal DNA repeats to generate extrachromosomal rDNA circles (18–21). Genetic studies indicate that *S. pombe* Rqh-1 is required to suppress inappropriate recombination that is essential for reversible S-phase arrest (22). Mutations in human *BLM*, responsible for Bloom Syndrome, are associated with a high frequency of sister chromatid exchange, sensitivity to several DNA damaging agents, and a defect in DNA replication (23). Finally, mutations in *WRN* result in large DNA deletions, possibly as a result of chromosomal rearrangements, a prolonged S-phase of DNA replication, and sensitivity to the genotoxic agent, 4-nitroquinoline-1-oxide (4, 5, 24–27). Together, these observations suggest that RecQ DNA helicases, including WRN, are involved in maintaining genomic stability, possibly by preventing deleterious recombination events from occurring during active DNA metabolism.

The BLM and WRN proteins are both human RecQ-like DNA helicases. Yet, mutations in the respective genes result in distinctly different phenotypes. Examination of the domain structure of the two proteins reveals that, outside the helicase domain, the two polypeptides share little, if any, homology (28). These regions, N- and C-terminal to the helicase domain, may encode other activities and/or interact with accessory proteins, both of which could determine the type of DNA transaction that each helicase carries out. Hence, identification of properties

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[1] The abbreviations used are: WS, Werner syndrome; BSA, bovine serum albumin; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

unique to each helicase may shed light on our understanding of their biological function(s). Recently, a conserved nuclease domain was identified in WRN by protein sequence data base searches (28–30). This domain is unique to WRN, spans amino acids 80–240 in the N terminus, and exhibits significant similarity to the 3′ → 5′ proofreading domain of *E. coli* DNA polymerase I, to RNaseD and to the nuclease domain of the human polymyositis/scleroderma nuclear autoantigen. Based on this homology, *WRN* was proposed to encode an exonuclease. In this report we present molecular genetic, biochemical, and immunochemical evidence to demonstrate that the WRN protein possesses an integral DNA exonuclease activity in addition to its intrinsic DNA helicase activity.

EXPERIMENTAL PROCEDURES

Materials and Enzymes—[γ-^{32}P]ATP was the product of NEN Life Science Products. High performance liquid chromatography purified oligodeoxynucleotides were provided by Operon Technologies. Bacteriophage T4 polynucleotide kinase was supplied by New England Biolabs. Leupeptin, aprotinin, phenylmethylsulfonyl fluoride, pepstatin, bovine serum albumin (BSA), dithiothreitol (DTT), Nonidet P-40, DEAE-cellulose, and protein A-agarose beads were purchased from Sigma. Cellulose phosphate (P-11) was provided by Whatman. His-Bind[2] affinity chromatography matrix and buffers were obtained from Novagen.

DNA Labeling and Annealing—Single-stranded DNA oligomers were 5′-end-labeled with ^{32}P according to Sambrook *et al.* (31) and were boiled immediately following labeling. To prepare the partial DNA duplex for helicase/exonuclease assays, the labeled oligomer was mixed with a 2-fold molar excess of a complementary unlabeled DNA oligomer in 50 mM Tris-HCl buffer, pH 8.0, 10 mM $MgCl_2$; following denaturation at 100 °C for 5 min, the DNA was allowed to anneal by slow cooling to room temperature.

DNA Helicase—DNA helicase activity was detected by the displacement of a ^{32}P-labeled 5′-20-mer oligonucleotide 5′-d(CGCTAGCAATATTCTGCAGC)-3′ from its partial duplex with the complementary unlabeled 46-mer 5′-d(GCGCGGAAGCTTGGCTGCAGAATATTGCTAGCGGGAATTCGGCGCG)-3′. Reaction mixtures contained in a final volume of 10 μl, 40 mM Tris-HCl buffer, pH 7.9, 4.0 mM $MgCl_2$, 5.0 mM DTT, 1.0 mM ATP, 10.0 μg of BSA, 0.1 pmol of labeled DNA substrate, and the indicated amounts of WRN. The reaction mixtures were incubated at 37 °C for 10 min, and DNA unwinding was terminated by rapid cooling on ice and by the addition of 2.0 μl of a solution of 2.0% SDS, 50 mM EDTA, 3.0% bromphenol blue, 3.0% xylene cyanol, 40% glycerol. The displaced single-stranded oligonucleotide was separated from the partial DNA duplex substrate by electrophoresis through a non-denaturing 12% polyacrylamide gel in TBE buffer (90 mM Tris base, 90 mM boric acid, 1.0 mM EDTA) at 4 °C for 2 h under 300 V (20 V/cm). Labeled DNA bands were visualized by autoradiography.

DNA Exonuclease—Hydrolysis of the 5′-^{32}P-labeled DNA oligomer by WRN exonuclease was conducted in the same reaction mixture used for the assay of DNA helicase activity except that the reaction was terminated by the addition of an equal volume of denaturing loading buffer. Products of DNA digestion were resolved by electrophoresis through a 14% polyacrylamide-urea gel (32) and visualized by autoradiography.

DNA-dependent ATPase—To determine DNA-dependent ATPase activity, WRN protein-catalyzed release of inorganic $^{32}PO_4$ from [γ-^{32}P]ATP was measured in the presence of DNA as described (12).

Expression of Recombinant WRN Proteins—*WRN* cDNA constructs were cloned into a recombinant baculovirus expression system (pBlueBacHis2A, Invitrogen) to yield full-length (GenBank™ accession number L76937) or truncated WRN proteins containing an N-terminal six-histidine residue tag. Control baculoviruses lacking WRN and viruses expressing WRN-WT or WRN-K577M were prepared as described previously (12). To generate WRN-ΔE, wild-type pBlueBacHis-WRN transfer vector was digested with *Bam*HI and *Bpu*1102I restriction endonucleases, and vector DNA lacking the N-terminal *WRN* cDNA fragment was gel-purified. The digested ends were filled and blunt-end ligated to create an in-frame fusion within the *WRN* cDNA excluding the exonuclease domain. This resulted in a deletion of amino acids 51–265 of the *WRN* coding sequence. To generate WRN-ΔH, a reverse transcriptase-polymerase chain reaction product spanning nucleotides 1145–3391 was generated from the WS cell line LGS90610 (which contains a nonsense mutation at codon 369 in *WRN*) (32) using the primers 5′-d(TTGAGACTGAACTGAGGCCCAG)-3′ and 5′-d(CTTTATGAAGCCAATTTCTACCC)-3′. The reverse transcriptase-polymerase chain reaction product was digested with *Bcl*I, and the fragment (nucleotides 1284–3296) containing the mutation was substituted for the corresponding wild-type *Bcl*I fragment in pBlueBacHis-WRN. Expression of this construct resulted in a C-terminal truncated WRN protein that lacks the helicase domain but retains the N-terminal nuclease domain. Recombinant baculoviruses were created by co-transfecting pBlueBacHis-WRN-ΔE or pBlueBacHis-WRN-ΔH transfer vectors with linearized wild-type AcMNPV viral DNA into *Spodoptera frugiperda* (Sf9) cells as described previously (12). Site-specific mutations, junctions of deletion constructs, and the coding region of the control virus were confirmed by sequencing.

Purification of WRN Helicase—Recombinant wild type WRN helicase bearing an N-terminal hexahistidine tag was expressed in Sf9 cells and purified by consecutive steps of ion exchange and Ni^{2+} affinity column chromatography. Frozen cells (packed volume 3.0–4.0 ml) were thawed and resuspended in 20 volumes of ice-cold extraction solution containing 20 mM Tris-HCl buffer, pH 7.9, 0.5 M NaCl, 0.5% Nonidet P-40, 10 μg/ml each of aprotinin, pepstatin, and leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, and 25% glycerol. All subsequent steps were conducted at 4 °C. The cells were disrupted in a teflon-glass homogenizer, the suspension was centrifuged for 15 min at 17,000 × *g,* and residual DNA was removed from the supernatant by chromatography through a DEAE-cellulose column equilibrated in buffer D (25 mM Tris-HCl buffer, pH 8.0, 0.5 mM EDTA, 0.05% Nonidet P-40, 1 mM DTT, 25% glycerol) supplemented with 0.5 M NaCl (33). Following overnight dialysis against ~ 50 volumes of buffer D, the extract, with a typical total protein content of 120–200 mg, was loaded onto a DEAE-cellulose column equilibrated in buffer D (5 mg of protein/ml of packed column volume). After washing the column with 1 volume of equilibration buffer, proteins were eluted by successive washes with three to four packed column volumes each of buffer D containing 100, 250, or 500 mM NaCl. SDS-PAGE resolution followed by Coomassie Blue staining of proteins indicated that the major portion of the ~160-kDa WRN protein was present in the 250 mM NaCl eluate. Pooled WRN fractions (typically 30–50 mg of protein) were dialyzed overnight against ~30 volumes of buffer P (50 mM KPO_4 buffer, pH 8.5, 50 mM DTT, 0.05% Nonidet P-40, 25% glycerol) and loaded onto a P-11 column, equilibrated in the same buffer (2.0 mg of protein/ml of packed column volume). Following wash with 1 column volume of equilibration buffer, a linear gradient consisting of 5 column volumes each of buffer P that contained 50 and 400 mM KPO_4, respectively, was applied to the column. Proteins eluted were collected into 40 fractions. The major portion of the ~160-kDa WRN protein as detected by SDS-PAGE and by measurement of its DNA helicase activity eluted from the P-11 column at 150–190 mM KPO_4. Pooled WRN protein fractions with a typical total protein content of 1.5–3.0 mg were dialyzed overnight against ~100 volumes of buffer N (20 mM Tris-HCl buffer, pH 7.9, 0.5 M NaCl, 0.05% Nonidet P-40, 25% glycerol) containing 5 mM imidazole and loaded onto a 1-ml His-Bind Ni^{2+} column equilibrated in the same buffer. Nonspecifically adsorbed proteins were washed with 8 column volumes of buffer N containing 30 mM imidazole. WRN protein, which remained tightly bound to the column, was eluted by a linear gradient of buffer N containing 30–300 mM imidazole (6 column volumes each). The eluate was collected into 40 fractions that were supplemented with 1 mM EDTA and 10 μg/ml each of leupeptin, aprotinin, and pepstatin. Eluted proteins were resolved by SDS-PAGE and assayed for activities of DNA helicase, DNA exonuclease, and DNA-dependent ATPase. These activities remained stable for at least 3 months at −80 °C when the WRN protein was supplemented with 100 μg/ml BSA and stored in buffer N containing up to 250 mM imidazole.

Immunoprecipitation of WRN—Polyclonal antibodies against wild type WRN protein were raised in a rabbit. The 160-kDa SDS-PAGE-resolved WRN protein was injected subcutaneously at ~0.1 μg of protein/g of body weight. This was followed by two booster injections of similar amounts of the purified protein administered at 3-week intervals. Immunoprecipitation of the helicase and exonuclease activities of WRN protein was conducted in a reaction mixture that contained in a final volume of 10 μl of buffer D, 0.1 mg/ml BSA, 8–100 μg of preimmune or immune serum, and 0.03 μg of WRN protein purified by a single step of His-Bind affinity chromatography. Following incubation at 4 °C for 1 h, 1.25 mg of swollen protein A-agarose beads were added and the mixture was agitated for 1, 2, or 4 h at 4 °C. Protein A-agarose beads and adsorbed immune complexes were removed by centrifugation at 6,000 × *g* for 5 min at 4 °C, and activities of DNA helicase and exonuclease were assayed in the supernatant fraction.

[2] His-Bind is the trade name for the metal chelation resin manufactured by Novagen.

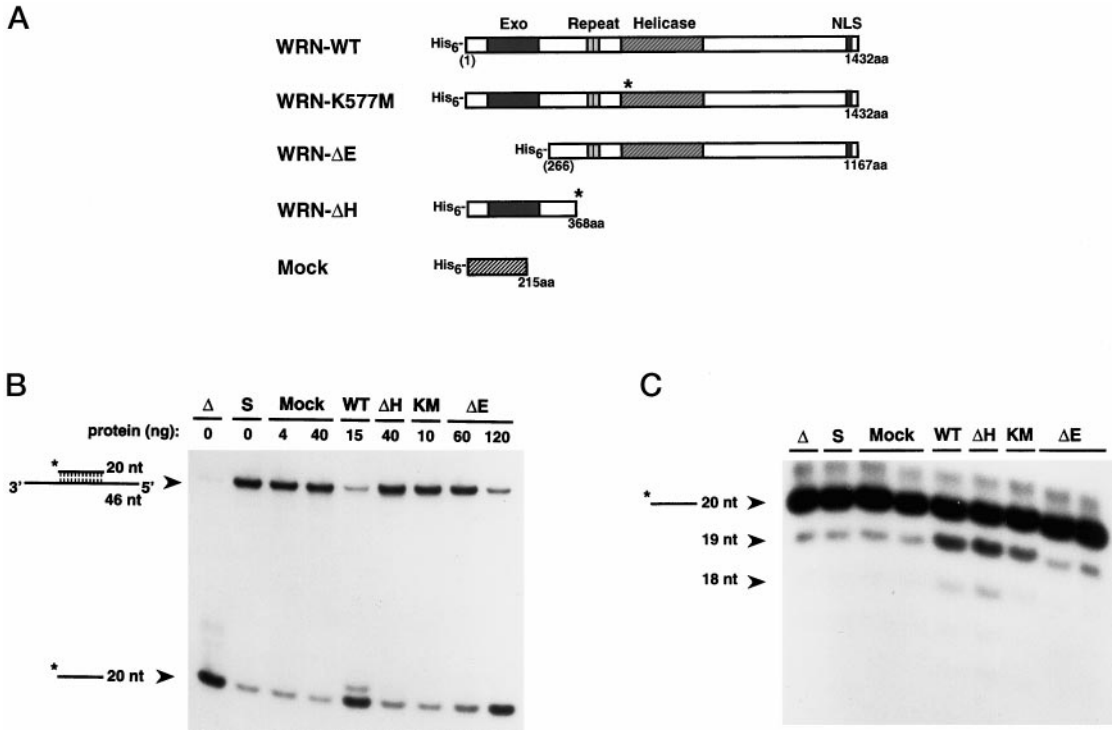

FIG. 1. **Helicase and exonuclease activities of wild-type and mutant WRN.** *A*, structures of the wild-type and mutant WRN constructs. Designations are as follows. *Exo*, putative exonuclease domain; *Repeat,* tandem repeat of 27 amino acids; *Helicase,* RecQ-like helicase domain; *NLS,* nuclear localization signal. *WRN-WT,* wild-type WRN; *WRN-K577M/KM,* mutant WRN with a lysine to methionine substitution at codon 577 (*) in helicase motif I (Walker ATPase A site); *WRN-ΔE,* mutant WRN containing N-terminal deletion that abolishes the predicted Exo domain; *WRN-ΔH,* mutant WRN with nonsense mutation at codon 369 that deletes the helicase and C-terminal NLS domains; *Mock,* non-functional His-tagged control peptide (lacking WRN) originating from the baculovirus expression vector. *B*, WRN helicase activity. Recombinant WRN and control proteins, indicated in *A*, were purified as described (12) and incubated with 0.1 pmol of ^{32}P-labeled 5′-20-mer/46-mer DNA substrate under standard helicase assay reaction conditions (see "Experimental Procedures"). Half of the reaction products were resolved by electrophoresis through a 12% polyacrylamide gel to visualize the displaced 5′-^{32}P-labeled 20-mer. *S,* DNA substrate; Δ, heat-denatured substrate; *, 5′-^{32}P label. *C,* WRN exonuclease activity. The remaining half of the reaction mixtures from *B* were resolved on a 14% polyacrylamide-urea gel as described.

RESULTS

DNA Helicase and Exonuclease Activities Are Encoded by Distinct Domains of the WRN Gene—An N-terminal domain in *WRN* was recently shown to encode a DNA sequence motif that is conserved in several exonucleases (28–30). To examine whether this domain encodes an active exonuclease, we expressed wild-type and mutant WRN proteins that either lack the putative N-terminal exonuclease domain or are defective in their helicase activities (Fig. 1*A*). The full-length and truncated WRN proteins were purified from baculovirus-infected insect cells by nickel chelation chromatography (12). The purity of all WRN preparations, except that of WRN-ΔE, was similar when visualized by SDS-polyacrylamide gel electrophoresis. Equivalent amounts of WRN from each preparation were assayed for helicase and exonuclease activities. Helicase activity was measured by the displacement of a ^{32}P-labeled 5′-20-mer from a partial duplex with a complementary unlabeled 46-mer oligonucleotide while exonuclease activity was monitored by the hydrolysis of the same substrate.

As shown in Fig. 1*B*, both WRN-WT and the WRN-ΔE mutant that lacks the exonuclease domain exhibited strand displacement activity. In contrast, WRN mutants lacking the central helicase domain (WRN-ΔH) or containing a mutation in the ATP-binding site (WRN-K577M or KM) did not exhibit detectable strand displacement activity. A control vector lacking WRN also did not possess helicase activity (Fig. 1*B*). Consistent with our previous observations (12), WRN-WT generated an additional band that migrated more slowly than the displaced 20-mer (Fig. 1*B*). This additional band was absent in reactions with WRN-ΔE, suggesting that the putative exonuclease domain was responsible for the generation of this band. Indeed, resolution of the reaction products on a denaturing gel (Fig. 1*C*) revealed that the band migrating more slowly on native gels was comprised of lower molecular weight degradation products. This ladder of more rapidly migrating oligonucleotides was observed when the labeled DNA substrate was incubated with WRN-WT, WRN-ΔH, and WRN-K577M and subjected to electrophoresis on denaturing gels. On the other hand, progressive shortening of the labeled oligonucleotides was neither observed in control reactions nor in mixtures incubated with WRN-ΔE. Hence, expression of nucleolytic activity by WRN depends on the presence of the N-terminal exonuclease domain.

Isolation of Highly Purified WRN Protein—To demonstrate biochemically that WRN protein possesses DNA exonuclease activity, it was purified extensively by a combination of ion exchange and affinity chromatography. SDS-PAGE analysis of progressively purified fractions of WRN protein shown in Fig. 2 indicates that although the ~160-kDa WRN polypeptide was detectable in Sf9 insect cell extracts, it was a minor component. Typically, 75% and 97% of total cellular proteins were removed by sequential chromatography on columns of DEAE-cellulose and P-11, respectively, resulting in an enrichment of WRN (Fig. 2). Binding of the P-11 fraction of WRN to a His-Bind column followed by elution with a linear gradient of imidazole resulted in further purification of the recombinant protein. SDS-PAGE and Coomassie Blue staining of proteins indicated that the ~160-kDa WRN polypeptide eluted from the His-Bind column was greater than 90% homogeneous. This preparation was used to examine the association of the exonuclease with the DNA helicase and DNA-dependent ATPase activities of the WRN protein.

Activities of DNA Helicase, Nuclease, and ATPase Co-purify with the WRN 160-kDa Polypeptide—The co-purification of a

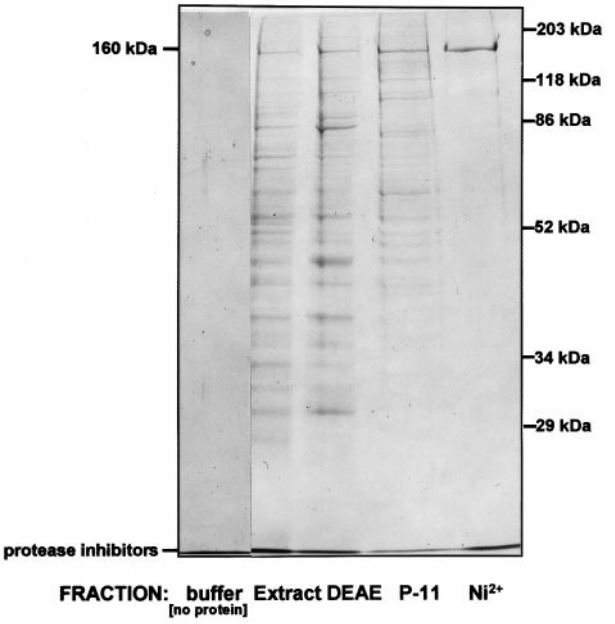

FIG. 2. **SDS-PAGE analysis of proteins in progressively purified fractions of WRN protein.** Aliquots (1.5 μg of protein each) of DNA-depleted Sf9 cell extract, fractions purified by DEAE-cellulose and cellulose phosphate, and the maximum allowable volume of Ni^{2+} column purified protein fraction were electrophoresed through an SDS-10% polyacrylamide gel, and the resolved protein bands were stained with Coomassie Blue. The protein concentration of each fraction was determined using the Bradford assay or by SDS-PAGE using known amounts of a standard BSA solution. Positions of molecular size marker proteins and of the 160-kDa WRN protein are indicated. The *left lane* was loaded with enzyme storage buffer containing 10 μg/ml each of pepstatin, leupeptin, and aprotinin that migrated at the forefront of the gel as indicated.

DNA exonucleolytic activity with the 160-kDa WRN polypeptide and its associated DNA helicase/ATPase activities was demonstrated by their overlapping elution patterns from a His-Bind column. The cellulose/phosphate-purified fraction of WRN protein was bound to a His-Bind column and eluted by a linear gradient of 30–300 mM imidazole. Whereas adsorbed proteins lacking the histidine tag were eluted from the column by 30 to ~90 mM imidazole, the 160-kDa WRN protein eluted only with 90–150 mM imidazole. A typical elution pattern of WRN protein and its associated activities is presented in Fig. 3. As shown, the amount of the 160-kDa polypeptide increased progressively with increasing concentrations of imidazole to reach a peak level in fractions 15–17 and then decreased. A highly overlapping elution pattern was observed for the activities of DNA helicase, exonuclease, and ATPase (Fig. 3). The co-elution of the helicase, exonuclease, and ATPase activities with the 160-kDa WRN polypeptide suggests that all three activities are catalyzed by the same high molecular weight polypeptide. To corroborate this conjecture, WRN protein obtained after a single step of His-Bind affinity chromatography was resolved by glycerol gradient centrifugation. As demonstrated in Fig. 4, the helicase and exonuclease activities co-migrated again in overlapping high molecular mass peaks thus behaving as high molecular size entities. Notably, these results negate the presence of contaminating low molecular weight nucleases in the WRN preparation.

Both DNA Helicase and Exonuclease Activities Are Immunoprecipitated by Antibodies against WRN Protein—Polyclonal rabbit antiserum against human WRN protein was used to demonstrate that the DNA exonuclease activity can be specifically co-immunoprecipitated with the DNA helicase activity. When used in an immunoblot analysis at a dilution of up to

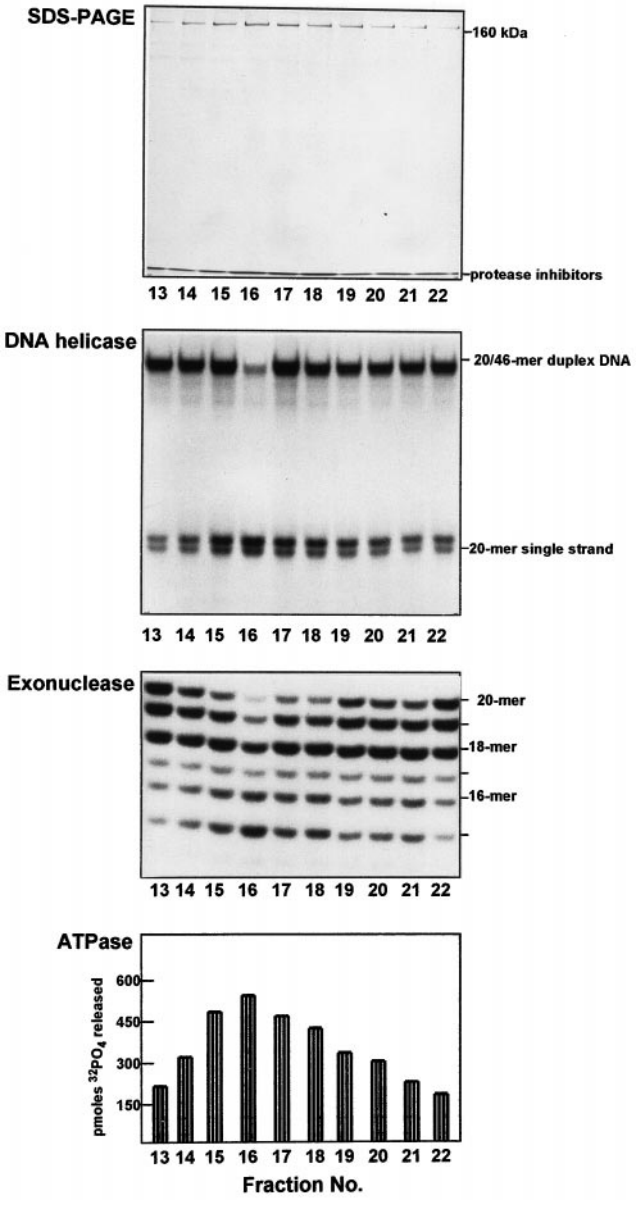

FIG. 3. **Elution pattern of WRN protein and its associated helicase, nuclease, and ATPase activities from a His-Bind Ni^{2+} column.** A cellulose/phosphate-purified fraction of WRN protein was chromatographed through a 1.0-ml Ni^{2+} column. WRN protein and its associated activities were eluted from the column by a linear gradient of 30–300 mM imidazole (see "Experimental Procedures"). *SDS-PAGE,* aliquots of Ni^{2+} column fractions were electrophoresed through an SDS-10% polyacrylamide gel, and proteins were stained with Coomassie Blue. Shown are fractions 13 to 22, the only ones that contained a detectable band of the 160-kDa WRN protein. *DNA helicase,* unwinding of a ^{32}P-labeled 5′-20-mer from its partial duplex with an unlabeled 46-mer was used to measure helicase activity in aliquots of the Ni^{2+} column protein fractions. Only fractions 13 to 22 exhibited measurable helicase activity. Positions of the partial DNA duplex and of the single-stranded 20-mer DNA are marked. *Exonuclease,* aliquots of reaction mixtures for the determination of DNA helicase activity were electrophoresed through a 14% polyacrylamide-urea gel to assess nucleolytic hydrolysis of the ^{32}P-labeled 5′-20-mer strand annealed to 46-mer DNA. Exonuclease activity was detected only in fractions 13 to 22 as shown. End-labeled marker oligonucleotides of known length were used to determine the length of the DNA fragments. *ATPase,* DNA-dependent ATPase activity was measured in fractions 13 to 22 as described (12).

10^{-4}, the antiserum specifically reacted with the 160-kDa WRN protein in insect cell extracts (data not shown). Recombinant wild-type WRN protein purified by a single step of His-Bind affinity chromatography was incubated with preim-

mune serum or with anti-WRN antiserum. Following adsorption of the immune complexes to protein A-agarose beads, activities of DNA helicase and DNA exonuclease were measured in the unadsorbed fraction (see "Experimental Procedures"). As shown in Fig. 5*A*, WRN DNA helicase activity in the supernatant remained undiminished following incubation with 10–100 μg of preimmune serum. A similarly unaffected activity of WRN exonuclease was observed after incubation with the preimmune serum (Fig. 5*C*). By contrast, incubation with 8–80 μg of anti-WRN antiserum followed by immune precipitation for 1 to 4 h diminished the activity of WRN DNA helicase in the supernatant by >90%, as visualized by the data in Fig. 5*B*. When an aliquot of the same reaction was electrophoresed on a denaturing gel, we observed that the major portion of the exonuclease activity was also immunoprecipitated by anti-WRN antibodies (Fig. 5*D*). These data further indicate that the two activities are encoded by the same antigenic WRN polypeptide.

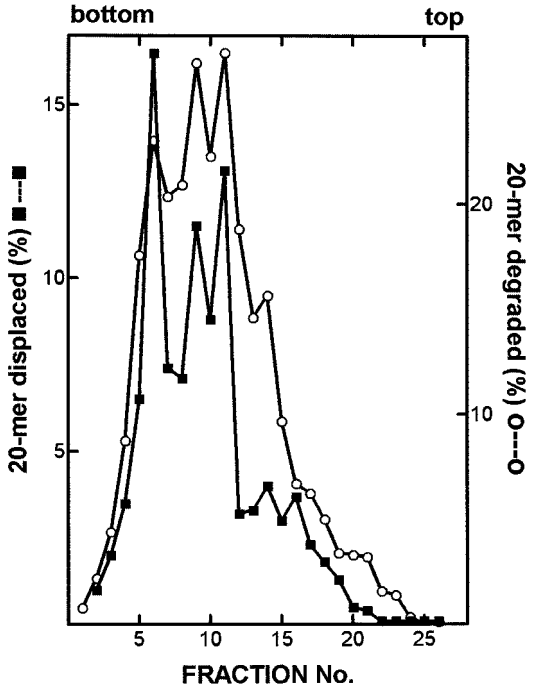

FIG. 4. **Glycerol gradient centrifugation of WRN DNA helicase and exonuclease.** Recombinant WRN protein purified by the single His-Bind affinity chromatography step was centrifuged through a 15–40% glycerol gradient in 25 mM Tris-HCl buffer, pH 8.0, 0.5 mM EDTA, 1 mM DTT, 25 mM NaCl for 25 h under 45,000 rpm (Sorvall 50.1 rotor) at 4 °C. Fractions (160 μl) were collected and assayed for activities of DNA helicase and exonuclease.

DISCUSSION

The recent identification in *WRN* of a nuclease domain with a sequence consensus similar to the 3′ → 5′ proofreading domain of *E. coli* polymerase I and to RNase D (28–30) led us to test whether WRN exhibits a deoxyribonucleolytic activity *in vitro*. Three complementary approaches independently indicate that *WRN* encodes an endogenous exonucleolytic activity in addition to a DNA helicase activity.

First we demonstrate a direct correlation between the presence of the putative WRN nuclease domain and observation of nuclease activity (Fig. 1). Full-length wild-type WRN purified by a single step of His-Bind affinity chromatography exhibits both DNA helicase and DNA exonuclease activities. Mutant proteins harboring either a deletion of the helicase domain (WRN-ΔH) or a single-base substitution (WRN-K577M) that inactivates ATP hydrolysis digest DNA exonucleolytically but fail to unwind it. In contrast, WRN-ΔE lacking the nuclease domain is unable to degrade DNA but retains its ability to displace DNA. Evidence for the presence of an exonuclease has also been obtained by Huang *et al*. (34). These authors site-

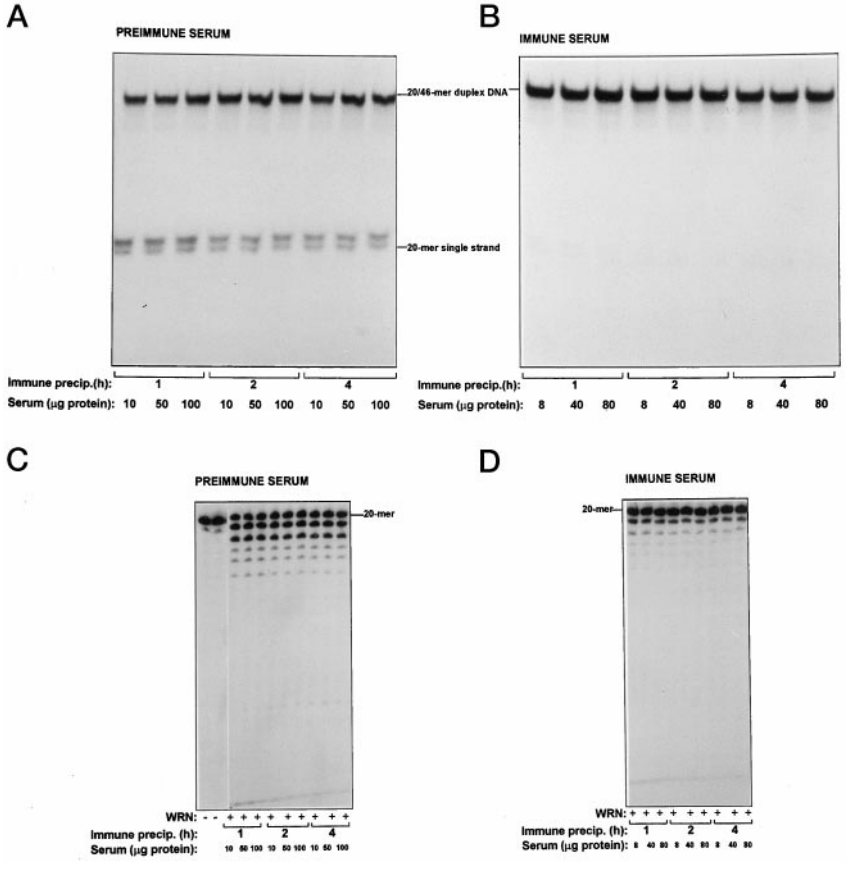

FIG. 5. **Immune precipitation of WRN DNA helicase and nuclease.** DNA helicase, WRN protein was incubated for 1 h at 4 °C with the indicated amounts of preimmune serum (*A*) or anti-WRN antiserum (*B*). Immune complexes were precipitated using protein A-agarose beads for the indicated periods of time at 4 °C. Following removal of the beads by centrifugation at 6,000 × *g* for 5 min, DNA helicase activity was assayed in the supernatant fractions as described in the legend to Fig. 1. DNA exonuclease, aliquots of the reaction mixtures from *A* and *B* were electrophoresed through a denaturing gel to resolve products of exonucleolytic digestion of the ^{32}P-labeled 5′-20-mer (*C* and *D*). Data presented in *panels C* and *D* were from the same autoradiogram developed after the same exposure times.

specifically altered two critical amino acid residues (Asp-82 and Glu-84) in the N-terminal exonuclease domain that are predicted to be important for nuclease activity. While the nuclease activity was abolished in these mutant proteins, the helicase activity remained unchanged relative to wild-type WRN. These genetic studies provide convincing evidence that hydrolysis of DNA is dependent on an intact N-terminal WRN exonuclease domain and that it does not require an intact helicase domain.

Second, our data provide biochemical evidence to suggest that the exonuclease and helicase activities reside on the WRN polypeptide. Both activities copurify with an extensively purified preparation of WRN. The elution profile of the exonuclease activity from the final step of His-Bind affinity chromatography is superimposable on the elution profiles of the 160-kDa WRN protein, the WRN DNA helicase and ATPase (Fig. 3). Furthermore, the WRN helicase and exonuclease activities co-migrate on glycerol gradients suggesting that both activities are encoded by a protein of a high molecular mass (Fig. 4). While most exonucleases are of low molecular weight, high molecular weight DNA polymerases frequently contain an associated 3′ → 5′ exonuclease that functions in proofreading errors by the polymerase (35). That the high molecular weight nuclease is not the result of such contaminating DNA polymerases was demonstrated by primer extension assays. No strand extension was observed when the DNA substrate used for helicase/exonuclease activity assays was incubated with WRN in the presence of all four dNTPs under DNA synthesis reaction conditions (not shown).

Finally, by incubating WRN with a polyclonal anti-WRN antiserum, we demonstrate that the helicase and nuclease activities are co-immunoprecipitated in a complex with protein A-agarose (Fig. 5). The depletion of the enzymatic activities of WRN is specific since neither activity is precipitated by the preimmune serum obtained from the same animal. These results further suggest that the helicase and exonuclease activities reside on the WRN polypeptide.

The presence of both a DNA helicase and a DNA exonuclease activity on the same polypeptide is so far unique to the WRN protein and may provide clues to its function in DNA metabolism. The fact that they are encoded within separate domains and that they can be uncoupled by selective deletion of each domain suggests that these activities need not function concertedly during the same catalytic step. Furthermore, studies with a mutant WRN protein in which the lysine residue (Lys-577) essential for NTP hydrolysis is substituted by a methionine residue show that the mutant protein can lose its ability to unwind DNA but retain its nuclease function. The spatial separation of helicase and exonuclease is reminiscent of many DNA polymerases in which polymerization and exonucleolytic hydrolysis occur sequentially (35). This could imply that the exonuclease functions in the removal of damaged DNA to yield a functional 3′-terminus suitable for elongation by a DNA polymerase that lacks proofreading exonucleolytic activity. Such a DNA polymerase presumably functions in concert with a 3′ → 5′ helicase that displaces duplex DNA ahead of the growing replication fork.

The ability of WRN helicase to exonucleolytically digest DNA also distinguishes it from other members of the RecQ helicase family. Although all members share the conserved helicase domains (7), none except WRN encodes an exonuclease domain. The presence of three, seemingly functionally redundant RecQ helicases in human cells (RecQL, BLM, and WRN) is puzzling. However, as more information is gained about possible other activities exhibited by these proteins, their substrate preferences, and their interaction with other proteins, they will likely be more distinct from one another. Hence, the helicase activity of WRN in conjunction with its exonuclease may limit the participation of WRN to processes that are distinct from those that require the RecQL and BLM proteins. Defective WRN activities could, therefore, impair these specific processes and result in the phenotypic manifestations that are characteristic of Werner Syndrome.

In summary, we have identified a novel exonuclease that is encoded within the N-terminal sequence of the WRN protein. In the following paper (36), we have biochemically characterized this activity and present evidence that it is a 3′ → 5′ exonuclease with properties distinct from other known exonucleases. The exonucleolytic activity exhibited by WRN could contribute to the unique characteristics of Werner Syndrome and distinguish it from other genomic instability disorders.

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