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# Biochemical Characterization of the WRN-1 RecQ Helicase of *Caenorhabditis elegans*†

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## Abstract

The highly conserved RecQ helicases are essential for the maintenance of genomic stability. Werner syndrome protein, WRN, is one of five human RecQ helicase homologues, and a deficiency of the protein causes a hereditary premature aging disorder that is characterized by genomic instability. A WRN orthologue, *wrn-1* lacking the exonuclease domain, has been identified in the nematode *Caenorhabditis elegans*. *wrn-1*(RNAi) in *C. elegans* has a shortened life span, increased sensitivity to DNA damage, and accelerated aging phenotypes. However, little is known about its enzymatic activity. We purified the recombinant *C. elegans* WRN-1 protein (CeWRN-1) and then investigated its substrate specificity in vitro to improve our understanding of its function in vivo. We found that CeWRN-1 is an ATP-dependent 3′—5′ helicase capable of unwinding a variety of DNA structures such as forked duplexes, Holliday junctions, bubble substrates, D-loops, and flap duplexes, and 3′-tailed duplex substrates. Distinctly, CeWRN-1 is able to unwind a long forked duplex compared to human WRN. Furthermore, CeWRN-1 helicase activity on a long DNA duplex is stimulated by *C. elegans* replication protein A (CeRPA) that is shown to interact with CeWRN-1 by a dot blot. The ability of CeWRN-1 to unwind these DNA structures may improve the access for DNA repair and replication proteins that are important for preventing the accumulation of abnormal structures, contributing to genomic stability.

Helicases make up a class of enzymes that separate nucleic acid duplexes into the constituent single strands. They play essential roles in DNA metabolic processes, including DNA replication, transcription, recombination, and repair (1). RecQ helicases, which are named after the DNA helicase RecQ found in *Escherichia coli*, have been shown to function in homologous recombination and repair of double-stranded breaks and are important for the maintenance of genomic stability (2,3).

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SUPPORTING INFORMATION AVAILABLE

DNA sequences of oligonucleotides (Table S1); unwinding of the blunt-ended duplex, effect of reaction parameters on CeWRN-1 helicase activity, effect of reaction temperature on CeWRN-1 helicase activity, and effect of reaction temperature on CeWRN-1 ATPase activity (Figure S1); alignment of amino acids in motif I of CeWRN-1 with human WRN and base sequence comparison between CeWRN-1 and the CeWRN-1-K255A mutant (Figure S2); EMSA of the 3′ overhang and the forked duplex substrates (Figure S3); and unwinding of D-loop substrates by CeWRN-1 (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

RecQ helicases are highly conserved from *E. coli* to humans, and several homologues of the helicases have been identified in various multicellular organisms (4). For example, five homologues have been found in humans to date: RECQ1, BLM (RECQ2), WRN (RECQ3), RTS (RECQ4), and RECQ5. Mutations in the genes that encode three of the human RecQ helicases, BLM, WRN, and RTS, have been associated with the hereditary disorders Bloom's syndrome (BS), Werner syndrome (WS), and Rothmund-Thomson syndrome (RTS), respectively (2,4). These syndromes are characterized by genomic instability at the cellular level, as well as hypersensitivity to agents that damage DNA.

To understand how the phenotypes of the human syndromes arise and to characterize the biological role of the RecQ helicases, researchers have studied the biochemical functions of the human RecQ helicases (5-8). The RecQ helicases unwind a wide variety of DNA structures, including flaps, D-loops, and Holliday junctions. These catalytic activities of the RecQ helicases suggest that they play important roles in processing aberrant DNA structures that arise during replication, repair, and recombination and provide evidence that the function of RecQ helicases is to maintain genomic stability. Because deficiencies of the RecQ helicases are associated with human inherited disorders, the functions of these helicases are likely to be important in the molecular pathology of such disorders (9-11). However, the precise DNA transactions that are mediated by the RecQ helicases remain elusive.

The human proteins WRN, BLM, RECQ1, *Drosophila* DmRECQ5*β*, yeast Sgs1, and *E. coli* RecQ all possess ATP-dependent 3′—5′ helicase activity (7,12-16). However, the recently characterized human RECQ4 has exhibited only ATPase activity (8). Uniquely, WRN has both 3′ → 5′ helicase activity and 3′ → 5′ exonuclease activity and can unwind many DNA structures (Holliday junctions, G4-quadruplex DNA, 12 nt[1] bubble structures, triple-helix structures, and forked structures), but it cannot unwind blunt-ended duplex DNA (5,14,17). Because these DNA structures are intermediates during recombination, repair, or replication, the DNA substrate specificity of WRN may confer genomic stability during these processes (9).

In the nematode *Caenorhabditis elegans*, four RecQ homologues are predicted from the genomic DNA sequence. The genes *recq1* (K02F3.12), *him-6* (T04A11.6), *wrn-1* (F18C5.2), and *recq5* (E03A3.2) are most homologous to the human RecQ helicase genes *RECQ1*, *BLM*, *WRN*, and *RECQ5*, respectively (4,18-21). Strains of *C. elegans* with *wrn-1*(RNAi) show premature aging phenotypes, such as a shortened life span and short stature, both of which are similar to those of human patients with WS (18). Premeiotic germ cells in *wrn-1*(RNAi) strains have an abnormal checkpoint response to blockage of DNA replication. These findings imply that WRN-1 may act as a checkpoint protein for DNA damage and blockage of replication (18). Deficiencies of RECQ5 and BLM in *C. elegans* also show a reduced life span and increased cellular sensitivity to DNA-damaging agents (22). However, the genomic instability found in human WS has not been reported in *C. elegans wrn-1* mutants or *wrn-1*(RNAi) strains, and little is known about the biochemical functions of any of the RecQ helicases of *C. elegans*.

To investigate the potential roles of the RecQ helicases of *C. elegans* in the maintenance of genomic stability, it is necessary to purify these proteins and characterize their activities biochemically. In this study, we have demonstrated that the *C. elegans* WRN-1 protein, CeWRN-1, possesses helicase activity and DNA-dependent ATPase activity. We have also shown that CeWRN-1 unwinds specific DNA structures that include 3′ and 5′ flaps, three-way junctions, a Holliday junction, D-loops, forked duplexes, and bubble substrates. CeRPA specifically stimulated the unwinding of long duplex DNA by CeWRN-1. To the best of our

[1]Abbreviations: DSB, double-stranded breaks; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; nt, nucleotide; ATP$\gamma$S, adenosine 5′-*O*-thiotriphosphate.

knowledge, this is the first demonstration of any DNA helicase in *C. elegans*. The implications of these activities of CeWRN-1 are discussed in relation to a potential cellular function for CeWRN-1.

## MATERIALS AND METHODS

### Bacterial Strains

*E. coli* BL21A1 [genotype, $F^-$ompT $hsdS_B$ ($r_B^-m_B^-$)gal dcm araB::T7RNAP-tetA] was used for expression of the recombinant CeWRN-1 protein.

### Recombinant DNA

The CeWRN-1 cDNA was cloned into expression vector pDEST17 for a six-His-tagged protein (Gateway Cloning System, Invitrogen, Carlsbad, CA). The PCR product of *C. elegans wrn-1* cDNA was first cloned into pENTR/D/TOPO. The sequence and orientation of the inserted *wrn-1* were confirmed by DNA sequencing (ABI 3730XL Capillary DNA Sequencer, GE Life Sciences, Piscataway, NJ). Subsequently, the cloned *wrn-1* in pENTR/D/TOPO was transferred to *E. coli* expression vector pDEST17 using LR Clonase. The cloned *wrn-1* was again confirmed by DNA sequencing. The pDEST17 containing *wrn-1* was then transformed into *E. coli* BL21A1 for protein expression.

### CeWRN-1 Protein Purification

The recombinant six-His CeWRN-1 fusion protein was expressed in *E. coli* BL21A1. The *E. coli* cells were grown at 37 °C in 2 L of LB medium containing 100 $\mu$g/mL ampicillin to an $OD_{600}$ of 0.4. L-Arabinose (Sigma-Aldrich, St. Louis, MO) was added to the culture to a final concentration of 0.2% (w/v) for induction, and the cells were then grown for an additional 4hat22 °C. Cultured cells were harvested by centrifugation, suspended in 40 mL of lysis buffer [50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.5 mM EDTA, 5 mM $\beta$-mercaptoethanol, 10% glycerol, 1 mM PMSF, protease inhibitor cocktail tablet (Roche, Mannheim, Germany), and 1 mM imidazole], and lysed by sonication (10 bursts at 10 s intervals). Lysates were clarified by centrifugation at 10000*g* for 30 min at 4 °C. The cleared lysates (10 mL) were mixed with 2 mL of 50% Ni-NTA agarose Superflow slurry (Qiagen GmbH, Hilden, Germany) and then incubated with rotary shaking at 4 °C for 1.5 h.

The mixture of lysate and Ni-NTA agarose was loaded onto a column. The column was washed with 10 column volumes of wash buffer 1 [50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.5 mM EDTA, 5 mM $\beta$-mercaptoethanol, 10% glycerol, and 10 mM imidazole], 10 column volumes of wash buffer 2 (wash buffer 1 but with 25 mM imidazole), and 5 column volumes of wash buffer 3 (wash buffer 1 but with 50 mM imidazole) to remove nonspecifically bound protein. CeWRN-1 was eluted with 5 mL of elution buffer (wash buffer 1 but with 300 mM imidazole). Peak fractions were dialyzed against buffer A [50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5 mM EDTA, 5 mM $\beta$-mercaptoethanol, and 10% glycerol], and the dialyzed proteins were loaded onto a Mono-Q column (Amersham, GE Life Sciences). The column was washed with 10 mL of buffer A and eluted with 10 mL of a linear gradient (from 50 to 500 mM) of NaCl in buffer B (buffer A containing 1 M NaCl). Fractions containing CeWRN-1 were detected using SDS-PAGE. The protein concentration was determined using a Bio-Rad assay with BSA as the standard. The purified CeWRN-1 protein was verified using a MALDI TOF-TOF 4700 proteomics analyzer (Postech Biotech Center, Pohang, Korea).

### Other Proteins

*C. elegans* WRN-1-K255A mutant and HA (influenza hemagglutin)-tagged *C. elegans* WRN-1 (CeWRN-1-HA) proteins were purified with the same method that was used for the CeWRN-1

protein. *C. elegans* RPA containing two subunits (large and middle subunits) was purified as described previously (23). *E. coli* SSB (single-strand binding) protein was purchased from Promega (Madison, WI).

## DNA Substrates

PAGE-purified oligonucleotides for DNA helicase substrates were synthesized by IDT (Coralville, IA) and are listed in Table S1 of the Supporting Information. For each substrate, a single oligonucleotide was labeled at the 5′ end with [$\gamma$-$^{32}$P]ATP (Amersham Radiochemicals, GE Life Sciences) using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) for 30 min at 37 °C and heat-inactivated for 10 min at 95 °C. To generate a series of helicase substrates (Table 1), the labeled oligonucleotide was annealed to its unlabeled complementary strand as described previously (5,24,25). The 100 bp M13mp18 partial duplex substrate was constructed using a 100 nt oligonucleotide (from IDT) complementary to positions 6039–6138 in the M13mp18 circular ssDNA (New England Biolabs).

## DNA Helicase Assay

Proteins and radiolabeled DNA substrates (10 fmol) were mixed in 10 $\mu$L of helicase reaction buffer [50 mM HEPES (pH 7.5), 20 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 2 mM dithiothreitol, and 0.1 mg/mL BSA]. Reaction mixtures were incubated at 37 °C for 15 min, and the reaction was terminated by the addition of 3× stop dye (0.05 M EDTA, 40% glycerol, 1% SDS, 0.05% bromophenol blue, and 0.05% xylene cyanol FF). Helicase reaction products were analyzed on nondenaturing 10% polyacrylamide gels (19:1 acrylamide:bisacrylamide ratio; Bio-Rad Laboratories, Hercules, CA). Radiolabeled DNA species in polyacrylamide gels were visualized using a phosphorimager (Typhoon 9400, GE Life Science) or Kodak Biomax MR Film (Eastman Kodak, Rochester, NY) and quantitated using ImageQuant (Molecular Dynamics, Sunnyvale, CA) or Scion image (Scion, Frederick, MD).

The percentage of unwound substrate was calculated using the following equation: % unwinding = 100[$P$/($S$ + $P$)], where $P$ is the intensity of the product and $S$ is the intensity of the substrate. The values of $P$ and $S$ were corrected by subtracting the background values obtained from the no enzyme and heat-denatured substrate controls, respectively. The helicase data represent the means of at least three independent experiments ± the standard deviation (SD).

## ATPase Assay

The ATPase assay reaction mixture (10 $\mu$L) contained DNA (50 ng for ssDNA, 7.4 kb) and CeWRN-1 (40 $\mu$M) or CeWRN-1-K255A (41 $\mu$M) in helicase reaction buffer without ATP. The reaction was initiated by the addition of ATP to a final concentration of 2 mM and the mixture incubated at 37 °C for 15 min. The reaction was terminated by adding 5.0 $\mu$L of 0.1 M EDTA (pH 8.0). The concentration of inorganic phosphate ($P_i$) released by ATP hydrolysis was measured using a colorimetric assay that was based on spectrophotometric quantification of a phosphomolybdate—malachite green complex (26). Briefly, 80 $\mu$L of the reaction sample was mixed in an ELISA microplate with 20 $\mu$L of malachite green reagent (Biosource, Camarillo, CA), whose limit of detection is 40 $\mu$M phosphate. The plates were scanned at 620 nm in a microplate ELISA reader (Wallac, Turku, Finland) after 5 min. The quantity of $P_i$ was then determined from a calibration curve derived from solutions with a known phosphate concentration ($KH_2PO_4$).

## Electrophoretic Mobility Shift Assay (EMSA)

Proteins were incubated with 5′-$^{32}$P-labeled duplex DNA substrates (10 fmol) for 15 min at 4 °C in 10 $\mu$L of helicase reaction buffer (no ATP), and the binding reaction mixtures were

subsequently analyzed on a 5% nondenaturing polyacrylamide gel in running buffer [20 mM Tris-HCl (pH 7.2), 10 mM NaOAc, and 0.5 mM EDTA] at 4 °C. Bands were visualized by autoradiography and quantitated using Scion Image.

### Dot Blotting

Indicated amounts of proteins were dotted on a polyvinylidene difluoride (PVDF) membrane prewetted with PBS and then incubated with blocking solution (1% fat-free milk with PBS-Tween 20) for 2 h at 4 °C. The membrane was incubated at 4 °C overnight with 1.5 $\mu$g of CeWRN-1-HA in blocking solution. After being washed, the membrane was incubated with mouse monoclonal anti-HA (Sigma-Aldrich) in blocking solution for 2 h at room temperature. Western blotting was performed using a horseradish peroxidase-conjugated secondary antibody and detected with an ECL kit (Amersham ECL, GE Life Sciences).

## RESULTS

### CeWRN-1 Protein Purification

To characterize the CeWRN-1 protein biochemically, CeWRN-1 was tagged with six histidines at its amino terminus using the Gateway cloning system. It was expressed in and then purified from *E. coli* using Ni-NTA agarose and Mono-Q columns to approximately 90% homogeneity (Figure 1A). The purified protein was analyzed by MALDI-TOF. As shown in Figure 1B, the purified protein clearly contained the amino acids encoded by the *C. elegans wrn-1* gene.

### CeWRN-1 Is a DNA 3′ → 5′ DNA Helicase

CeWRN-1 contains conserved helicase motifs of the RecQ helicase family (20,27) and therefore is expected to have DNA unwinding activity. To determine whether CeWRN-1 possesses DNA helicase activity, we first tested its ability to disrupt four different DNA substrates (5′ ssDNA tail, 3′ ssDNA tail, forked duplex, and blunt-ended duplex substrates). The substrates were generated following the annealing of combinations of the oligonucleotides (Table 1 and Table S1). Single oligonucleotides were 5′-end-labeled with [$\gamma$-$^{32}$P]ATP as indicated in Table 1. Helicase reaction mixtures contained 1 nM DNA substrates and increasing amounts of CeWRN-1. Displacement of $^{32}$P-labeled oligonucleotides was monitored by nondenaturing polyacrylamide gel electrophoresis and analyzed using autoradiography. The percentage of DNA unwinding was calculated.

We observed that CeWRN-1 displaced 3′-tailed and forked duplexes efficiently in a concentration-dependent manner (Figure 2A–D). At a protein concentration of approximately 5–10 nM, 50% strand displacement occurred in 3′-tailed and a short forked duplex (Figure 2E). However, CeWRN-1 was ineffective at unwinding DNA substrates with a 5′ ssDNA tail or blunt ends (Figure 2C and Figure S1-A of the Supporting Information). This indicates that CeWRN-1 binds to the 3′ ssDNA tail, initiates unwinding, and translocates along the bound ssDNA in a 3′ to 5′ direction, resulting in the displacement of the two strands of the duplex region. Because the polarity of movement of a DNA helicase is defined as the direction of translocation along the bound strand, this result indicates that CeWRN-1 is a 3′ to 5′ helicase, which is a general characteristic of the RecQ helicase family (7, 14, 15, 28). CeWRN-1, at lower concentrations, unwound the short forked duplex to a greater extent than the 3′-tailed duplex. This suggests that CeWRN-1 is more efficient at unwinding a forked duplex than a tailed duplex.

It has been shown that human WRN helicase and exonuclease act in coordination to remove a DNA strand from a long forked duplex (for example, a 34 bp forked duplex) that is not unwound by the helicase alone (29). Digestion at the blunt end shortens the duplex so that helicase can fully unwind it. Since CeWRN-1 does not have an exonuclease domain, whether CeWRN-1

unwinds a long forked duplex is of interest. Figure 2D shows that CeWRN-1 unwound a 34 bp forked duplex without digestion of the blunt end because the resolved products of the long forked duplex comigrated with the heat-denatured DNA substrate control (lane 7 in Figure 2D). However, the unwinding of the long forked duplex was less efficient than that of short forked and 3′-tailed duplexes.

We examined parameters that affect CeWRN-1 helicase activity (Figure S1-B). Little or no unwinding was observed when $Mg^{2+}$ was replaced with $Zn^{2+}$. The CeWRN-1 helicase was active in reaction buffer containing 50–200 mM KCl and in a pH range from 6.5 to 8.0. In addition, CeWRN-1 helicase activity was compared at different temperatures, 20, 30, and 37 °C for 15 and 30 min incubations (Figure S1-C). The CeWRN-1 was similarly active at these three temperatures.

## CeWRN-1 Helicase Is Coupled to Its DNA-Dependent ATPase

Because CeWRN-1 contains motifs I and II of the RecQ helicase family, which are required for ATP binding and hydrolysis (27), we investigated whether the DNA unwinding activity of CeWRN-1 is dependent on ATP hydrolysis. When the poorly hydrolyzable ATP analogue ATP$\gamma$S was used in helicase reactions with the 3′-tailed DNA substrate and CeWRN-1, strand displacement was not observed (Figure 3A). This indicates that ATP hydrolysis is required for CeWRN-1 to unwind duplex DNA.

The invariant lysine residue in motif I of the RecQ helicases, which is also known as the Walker A-type motif (TGxG**K**S, Figure S2-A), has been demonstrated to be important for ATPhydrolysis and DNA unwinding (14,30,31). We replaced the lysine residue (K255) of CeWRN-1 with an alanine residue (Figure S2-B). CeWRN-1-K255A proteins were purified in a manner identical to that of the wild-type CeWRN-1 protein, and helicase assays were then conducted on the 3′ ssDNA tailed duplex substrate. Wild-type CeWRN-1 unwound the substrates, whereas no unwinding of the partial duplex DNA substrate by CeWRN-1-K255A was detected (Figure 3C). This indicates that the K255A substitution abolished the unwinding function of CeWRN-1 and shows that this lysine residue of CeWRN-1 is important for DNA unwinding.

Having shown that ATP hydrolysis and the invariant lysine residue were required for the unwinding activity of CeWRN-1, we examined CeWRN-1 for ATPase activity. Reactions were carried out either in the absence of DNA or in the presence of DNA effectors. The level of ATP hydrolysis was measured by the malachite green phosphate assay. CeWRN-1 was found to exhibit ATPase activity in the presence of DNA (Figure 3B). Both ssDNA and dsDNA stimulated ATP hydrolysis by wild-type CeWRN-1, but only very weak ATPase activity was observed when DNA was omitted (Figure 3B, white symbols). The ssDNA was much more efficient than the dsDNA in stimulating CeWRN-1 ATPase activity. The CeWRN-1-K255A mutant lost the ability to hydrolyze ATP (Figure 3B, black symbols), thus supporting the possibility that ATP hydrolysis is coupled to the unwinding activity of CeWRN-1. Similarly, a mutation in the invariant lysine residue of conserved motif I (the Walker A ATPase site) of the human WRN-K577M (Figure S2-A) protein abolished its helicase and ATP hydrolysis activities (14). Together, these results indicate that CeWRN-1 has DNA-dependent ATPase activity similar to that of other members of the RecQ helicase family.

Because the wild-type and CeWRN-1 mutant proteins were also purified using the same method, the results shown in Figures 2 and 3 indicate that the observed helicase and ATPase activities are truly dependent on CeWRN-1 and are not due to contamination that may have occurred during the preparation of CeWRN-1.

### Unwinding of DNA Recombination Intermediates

Because human RecQ helicases can unwind potentially recombinogenic DNA structures that include four-way junctions (which mimic Holliday junctions) and D-loops, we investigated whether CeWRN-1 can catalyze similar reactions on these substrates.

CeWRN-1 was able to unwind a Holliday junction to produce a single-stranded oligonucleotide and a forked duplex in the presence of ATP (Figures 4A and4B). The appearance of a forked duplex suggests the product of branch migration on a Holliday junction (Figure 4B). Comparatively, few three-strand junctions were observed.

CeWRN-1 was also able to unwind all three D-loop substrates in a concentration-dependent manner in helicase reactions (Figure S4).

### CeWRN-1 Unwinds Synthetic Replication Fork Intermediates

The two RecQ helicases, WRN and BLM, have exhibited unwinding of three-way junction, 5′ ssDNA flap, and 3′ ssDNA flap substrates. These substrates are particularly relevant in a biological context (5,6,32). A three-way junction resembles a stalled replication fork in which the forked duplex contains both 5′ dsDNA and 3′ dsDNA tails (Table 1). A flap substrate is a dsDNA substrate that has a ssDNA tail flanked immediately by an upstream DNA duplex (Table 1), which would be generated at a stalled replication fork in which DNA synthesis of the lagging or leading strand has been blocked (15).

We investigated unwinding of these three substrates by CeWRN-1 and tested whether CeWRN-1 can unwind dsDNA substrates that possess a junction but lack either one or both of the preexisting ssDNA tracts that flank the duplex region. A typical CeWRN-1 helicase assay was conducted with the substrates radiolabeled at the 5′ end (Figure 5). CeWRN-1 unwound the 5′ flap substrate to release the labeled 5′ flap oligonucleotide, as evidenced by its comigration with the heat-denatured substrate control (Figure 5A). This observation indicates that CeWRN-1 does not require a preexisting free 3′ ssDNA tail adjacent to the duplex region to unwind the substrate and that it may be able to unwind dsDNA substrates that possess a junction.

We tested the 3′ flap substrate, which is a forked substrate with a 3′ ssDNA tail and a 5′ dsDNA tail. A partial duplex structure was a major reaction product (Figure 5B), and less than 10% of all the reaction products were a 5′-labeled oligonucleotide (lane 3 in Figure 5B). Because CeWRN-1 exhibits 3′ to 5′ polarity, like other RecQ helicases, CeWRN-1 can bind to the 3′ ssDNA tail and then translocate in a 3′ to 5′ direction, resulting in the production of a partial duplex. This observation indicates that CeWRN-1 does not require the 5′ ssDNA element of the forked duplex to unwind the substrate. However, the possibility that CeWRN-1 helicase may act upon the upstream annealed primer to unwind the oligonucleotide, thereby generating a forked duplex that would be unwound by CeWRN-1, cannot be ruled out.

We also tested a three-way junction substrate, which is a forked duplex that mimics a synthetic replication fork (Figure 5C). A major unwound product of this substrate was a partial duplex structure, and this finding indicates that the CeWRN-1 helicase specifically unwinds in the direction of the synthetic replication fork, thereby separating the two template strands. This result suggests that CeWRN-1 can recognize the junction of forked duplexes that lack ssDNA.

### CeWRN-1 Unwinds Bubble Structures

No unwinding of a blunt duplex substrate of 19 bp indicates that CeWRN-1 cannot initiate unwinding from blunt ends or internal duplex regions (Figure S1-A). However, given that CeWRN-1 was able to unwind fork, flap, and D-loop structures that contained ssDNA regions

(Figures 4 and 5), we were interested in an internal single-stranded entry site. Thus, we tested the unwinding of bubble substrates that contained different single-stranded regions of 4, 12, and 21 nt (Figure 6). The 4 nt bubble substrate was not unwound (Figure 7A), which suggests that the protein cannot load at the ssDNA–duplex DNA junction provided by the 4 nt bubble. However, increasing the size of the bubble dramatically improved the ability of CeWRN-1 to use this structure as a substrate for unwinding (Figure 6B,C), which indicates that CeWRN-1 is able to recognize the ssDNA–dsDNA junction provided by 12 or 21 nt bubbles.

### Unwinding of CeWRN-1 on a Long Partial DNA Duplex Requires Replication Protein A (RPA)

We analyzed the helicase activity of CeWRN-1 on a long partial DNA duplex substrate in a 100-mer annealed to ssM13mp18 DNA and found that CeWRN-1 was unable to displace this oligonucleotide significantly.

Because human RPA has been shown to enhance specifically the unwinding of long DNA duplexes by WRN and BLM (24), we investigated the effect of the CeRPA, a *C. elegans* ssDNA-binding protein, on unwinding of a long DNA duplex (Figure 7). We observed that the addition of CeRPA greatly stimulated unwinding of the M13-based long partial duplex by CeWRN-1 in a concentration-dependent manner, but ESSB protein (*E. coli* SSB protein) did not stimulate the unwinding of this substrate (Figure 7A,B). In control reactions, CeRPA (118 nM, heterodimer) or ESSB protein (368 nM, homotetramer) alone did not displace. Because it has been shown that a physical interaction between human WRN and hRPA plays an important role in the mechanism of RPA stimulation of long DNA duplex unwinding (24), we investigated whether CeWRN-1 interacts with CeRPA. Dot blotting was performed with purified proteins. CeWRN-1 strongly bound to CeRPA, but no interaction was detected with the ESSB protein or BSA (Figure 7C), suggesting that CeWRN-1 specifically interacted with CeRPA.

## DISCUSSION

There are four RecQ protein homologues in *C. elegans*. However, the biochemical functions of the RecQ proteins have not been investigated to decipher their mechanisms of action. Since *wrn-1*(RNAi) in *C. elegans* has a shortened life span, increased sensitivity to DNA damage, and accelerated aging phenotypes, the function of CeWRN-1 may contribute to an understanding of its phenotypes. In this study, we have successfully expressed a full-length *C. elegans* WRN-1 (CeWRN-1) protein in *E. coli*, purified it to near homogeneity, and examined its enzymatic activities.

For its initial biochemical characterization, we investigated whether CeWRN-1 has helicase activity. Our studies have revealed that CeWRN-1 was able to unwind 3′ ssDNA tailed duplex and forked duplex substrates but did not unwind 5′ ssDNA tailed duplex and blunt-ended duplex substrates (Figure 2). This indicates that CeWRN-1 is a 3′ → 5′ helicase, a characteristic that is shared by other members of the RecQ protein family that have been studied to date (4).

The more efficient unwinding of the short forked duplex (Figure 2E) versus that of a 3′-tailed duplex substrate suggests that CeWRN-1 binds efficiently to and initiates unwinding from the junction on the forked duplex substrate. This finding is consistent with previous reports in which human WRN and BLM prefer a forked duplex to a duplex with a ssDNA tail for unwinding (5, 32). To test this possibility, EMSAs were performed with radiolabeled substrates and CeWRN-1 (Figure S3). More complexes were formed with the forked duplex substrate than with the 3′ overhang (Figure S3). This result suggests that CeWRN-1 preferentially binds to a forked duplex rather than to a simpler substrate with a 3′ ssDNA tail and implies that this binding preference is important in the efficient unwinding of the DNA substrate.

In this study, we showed that CeWRN-1 was able to unwind a 34 bp forked duplex. This ability of CeWRN-1 is different from that of human WRN. The human WRN could not sufficiently unwind the same size forked duplex (29). However, this long forked duplex was unwound in concert with the exonuclease domain of human WRN, resulting in shorter products. This evidence showed that the helicase and the exonuclease domains act coordinately in the full-length protein. A sequence alignment of human WRN and CeWRN-1 showed that CeWRN-1 lacks an exonuclease domain at the N-terminus (18). Thus, CeWRN-1 may be able to unwind long forked duplexes without a coordination of exonuclease activity in a full-length protein. Interestingly, *C. elegans* has a human exonuclease homologue protein. Although the activity has not been identified, the regulation of this protein with respect to CeWRN-1 remains to be assessed in further studies.

The unwinding efficiency of the long forked duplex is lower than those of short forked and 3′-tailed duplexes. Since the binding efficiency with respect to a long forked duplex is similar to that of a short forked duplex (data not shown), low unwinding efficiency may be due to weak processive action of CeWRN-1.

The ATPase activities of other RecQ helicases have been reported previously (8,14,20,27, 31). A comparison of conserved motifs I and II in other RecQ family members with CeWRN-1 suggested that CeWRN-1 would have ATPase activity. Our results revealed that CeWRN-1 possesses DNA-dependent ATPase activity (Figure 3B), consistent with other RecQ helicases such as human WRN, RecQ4, RecQ1, and BLM. When the poorly hydrolyzable ATP analogue ATP$\gamma$S was used in a helicase reaction, a 3′-tailed duplex substrate was not unwound (Figure 3A), indicating that CeWRN-1 requires energy from ATP hydrolysis for unwinding of duplex DNA. In addition, mutation of the invariant lysine residue (K255) in the conserved TGXG**K**S sequence (RecQ helicase motif I) in CeWRN-1 resulted in the loss of its helicase and ATPase activities (Figure 3B,C). Thus, the conserved lysine residue is important for ATP hydrolysis and unwinding of DNA, further demonstrating that the helicase activity of CeWRN-1 is coupled to ATP hydrolysis. Consistent with these results, loss of ATPase and helicase activities has also been observed in the human WRN-K577M mutant (14,30).

Although the recombinant CeWRN-1 protein was expressed at 22 °C, the purified protein exhibited active helicase activities at different temperatures (20, 30, and 37 °C), indicating that the purified CeWRN-1 remains active at different temperatures. However, since the temperature range for *C. elegans* living is from 15 to 25 °C, the kinetics of ATPase or helicase may show a difference to some extent. The kinetics of ATPase was examined at 22 and 37 °C (Figure S1-D). The stimulation of ATPase by DNA was more efficient at 37 °C than at 22 °C.

RecQ helicases have been shown to unwind a wide range of DNA structures (5,14,15,32,33). Here, CeWRN-1 has been shown to unwind various DNA structures (Table 1). Because the structures unwound by CeWRN-1 show overlap with structures unwound by human WRN and BLM, CeWRN-1 may function similarly in cellular DNA metabolism and may be involved in mechanisms such as recombination, replication, and repair in which WRN and BLM have been proposed to function (4,9,10). In addition, our results indicate that CeWRN-1 has the ability to recognize certain structural elements of DNA that are likely to be important for efficient loading onto these DNA structures and the initiation of unwinding.

The finding that CeWRN-1 unwinds a 3′ ssDNA tailed duplex substrate indicates that CeWRN-1 requires a free 3′ ssDNA tail to initiate unwinding of duplex DNA substrates. However, the finding that CeWRN-1 has the ability to unwind 5′ flap and three-way junction (resembling a synthetic replication fork) substrates also suggests that CeWRN-1 has the ability to recognize a junction of duplex DNA substrates, a ssDNA—dsDNA junction or a dsDNA—dsDNA junction, to initiate DNA unwinding efficiently but does not require a preexisting 3′

ssDNA tail in a helicase substrate for either loading or initiation of unwinding (Figure 5A,C). Thus, CeWRN-1 may use two distinct modes to initiate DNA unwinding, depending on the type of DNA structure it encounters.

The action of the CeWRN-1 helicase on the 5′ flap substrate suggests that CeWRN-1 helicase may regulate the size of the 5′ ssDNA flap of the Okazaki fragment with the coordinated displacement synthesis by DNA polymerase, as proposed for human WRN or the 5′ ssDNA flap during DNA synthesis in long-patch base excision repair by a DNA polymerase (25). In addition, because the 5′ flap structure can be envisioned as a stalled replication fork in which synthesis of the lagging strand has been blocked, CeWRN-1 may be involved in the recovery of stalled replication forks.

The predominant production by CeWRN-1 of a partial duplex from a three-way junction substrate (Figure 5C) demonstrates that CeWRN-1 recognizes the junction of the substrate and unwinds specifically in the direction of the fork. Because CeWRN-1 retains a 3′ → 5′ directionality of movement during unwinding, CeWRN-1 translocates along the lagging strand template in a 3′ → 5′ direction, separating the two template strands ahead of the fork structure. This unwinding specificity is identical to that of the human WRN helicase but is different from that of *Drosophila* dmRECQ5, which unwinds 5′ flap substrates inefficiently and unwinds in an outward direction from the fork (15).

CeWRN-1 was able to catalyze unwinding of a Holliday junction (Figure 4). Because a two-way junction (effectively a forked structure) was detected as a product of unwinding of a Holliday junction, it is possible for CeWRN-1 to disrupt the Holliday junction by promoting branch migration. Subsequently, CeWRN-1 unwinds the resulting forked structure to produce single-stranded oligonucleotide products.

Because a Holliday junction is an intermediate formed during a homologous DNA recombination process and can be induced by replication fork regression (reversal) at a blocked fork (34,35), the unwinding of a Holliday junction by CeWRN-1 demonstrates that CeWRN-1 may participate in the processing of recombination intermediates to prevent aberrant recombination events during homologous recombination and/or the restarting of replication forks as proposed for the RecQ helicases BLM and WRN.

In addition, this observation indicates that CeWRN-1 can utilize the internal structural features of DNA to recognize and initiate unwinding and supports the hypothesis that a 3′ ssDNA tail is not a structural requirement for the unwinding of duplex DNA by CeWRN-1.

CeWRN-1 was able to displace catalytically the invading strand of D-loop substrates regardless of the presence of ssDNA tails (Figure S4). Since D-loop intermediates arise from homologous recombination as a result of double-stranded breaks, CeWRN-1 may be involved in the homologous recombination process.

We found that CeRPA stimulated unwinding of a long partial DNA duplex by the helicase activity of CeWRN-1. The enhancement of CeWRN-1-catalyzed strand displacement is specific for CeRPA because CeRPA performed significantly better than the ESSB protein (Figure 7A,B). Such similar specific stimulation of WRN by hRPA was also found (24, 30). A specific interaction between CeWRN-1 and CeRPA may play a role in this stimulation (Figure 7C). Mechanistically, SSB proteins could bind to displaced ssDNAs during helicase reactions and prevent the displaced ssDNAs from annealing with the DNA template. Conversely, if there is no specific coordination between the two proteins, certain high concentrations of SSB protein might inhibit strand displacement by competing with the helicase for binding at the junction of single- and double-stranded DNA. Thus, this finding indicates

that CeWRN-1 and CeRPA coordinately unwind long DNA duplexes in vitro. However, in vivo interaction between CeWRN-1 and CeRPA remains to be investigated.

*C. elegans wrn-1*(RNAi) strains have shortened life spans and an accelerated S-phase in their early stages of development (18). When such strains are irradiated with ionizing radiation, the frequency of *wrn-1*(RNAi) aging phenotypes is increased significantly. In addition, *wrn-1* (RNAi) strains treated with hydroxyurea show small nuclei in the gonad. These phenotypes indicate that CeWRN-1 may play a role in activating the checkpoint for blockage of DNA replication in the repair of damage-induced lesions or may be active at stalled replication forks that arise from DNA damage (18).

The similarity of the substrate specificity of CeWRN-1 helicase to that of human WRN leads us to suggest that CeWRN-1 may act on specific substrates that arise in recombinational repair pathways and/or during the recovery of stalled replication forks. Alternatively, the ability of CeWRN-1 to unwind duplex and alternative DNA structures may increase the access for repair and replication proteins that are important for preventing the accumulation of abnormal structures, thus conferring genomic stability. For this possibility, the localization of CeWRN-1 in response to DNA damage or replication blockage remains to be examined.

To date, however, there is little information about the genomic instability of the phenotype exhibited by *wrn-1* deficiency available. This makes it particularly difficult to identify a biochemical function for CeWRN-1 in cellular DNA metabolism. Thus, further analysis of the precise roles of CeWRN-1 is particularly important in shedding light on the functions of the CeWRN-1 helicase in the maintenance of genomic stability.

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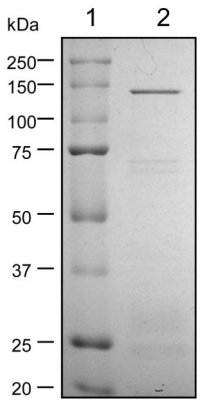
A




B

| Peptide | AA position | E value |
|---|---|---|
| TENQVADEDYDSFDDFVPSQTHTASK | 50 | 1e-16 |
| NVLGGKDQFVLMSTGYGK | 238 | 2e-08 |
| NSQQKEEVVENLTMMLR | 565 | 1e-08 |
| IREYEPANENEQLMNLKK | 782 | 1e-09 |
| GISEGTVYSYLAMAVEK | 933 | 1e-07 |

**Figure 1. Purification of CeWRN-1**

(A) SDS–PAGE analysis of purified CeWRN-1. CeWRN-1 was overproduced in *E. coli* as a fusion with an N-terminal six-histidine tag. Purified six-His-tagged CeWRN-1 (1 $\mu$g in lane 2) was analyzed by 8% SDS—PAGE and stained with Coomassie Blue. Lane 1 contained molecular size markers. (B) Five representative fragments (of 27 identified in total) of purified CeWRN-1 analyzed by MALDI-TOF. Amino acid (AA) positions are as indicated, and their corresponding *E* values are listed.

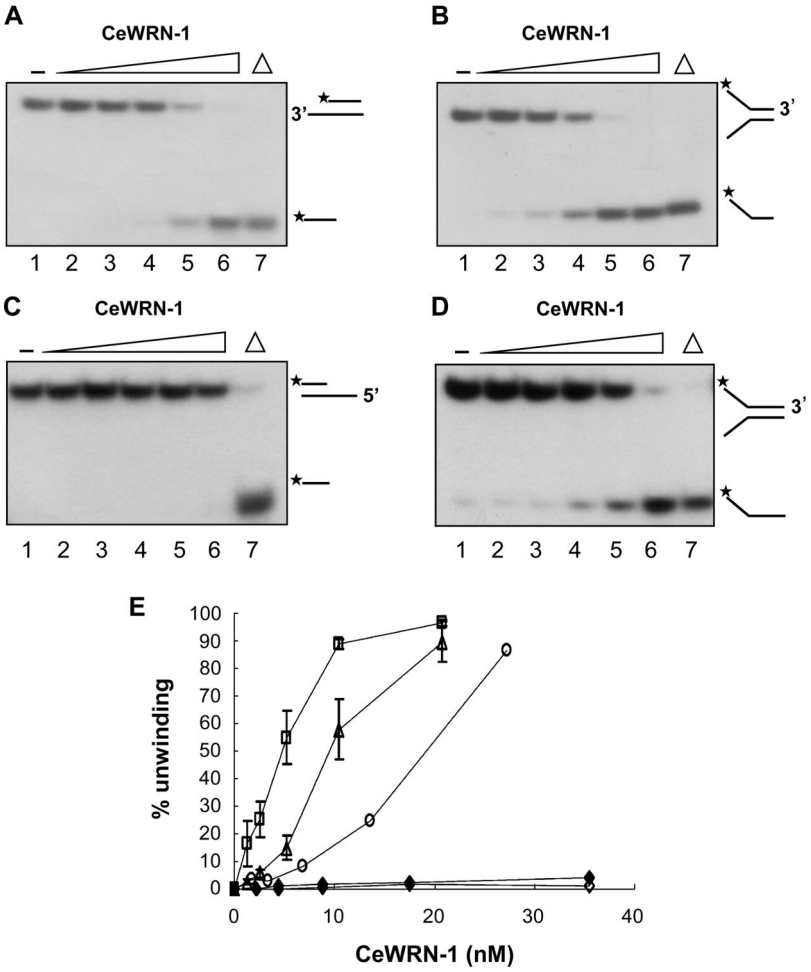



**Figure 2. Helicase activity and polarity of CeWRN-1**

Substrates were (A) the 3′ ssDNA tailed duplex, (B) a short forked duplex, (C) the 5′ ssDNA tailed duplex, and (D) a 34 bp forked duplex. Helicase reactions were carried out as described in Materials and Methods: lane 1, no enzyme control; lane 7, heat-denatured DNA substrate. Lanes 2–6 show a protein titration (1.5, 3.0, 6.0, 12, and 24 nM for panels A and B; 2.2, 4.4, 8.8, 17.6, and 35.2 nM for panel C; and 1.7, 3.4, 6.8, 13.6, and 27.2 nM for panel D). The substrates and products are shown at the right. The asterisk denotes the $^{32}$P label. (E) Quantitation of the data from panels A–D and Figure S1 of the Supporting Information. The percentage unwinding is expressed as described in Materials and Methods. Data represent the means of at least three independent experiments ± SD: (□) a 22 bp fork, (△) the 3′ ssDNA tailed duplex, (◆) the 5′ ssDNA tail, (◇) the blunt-ended duplex, and (○) a 34 bp fork.

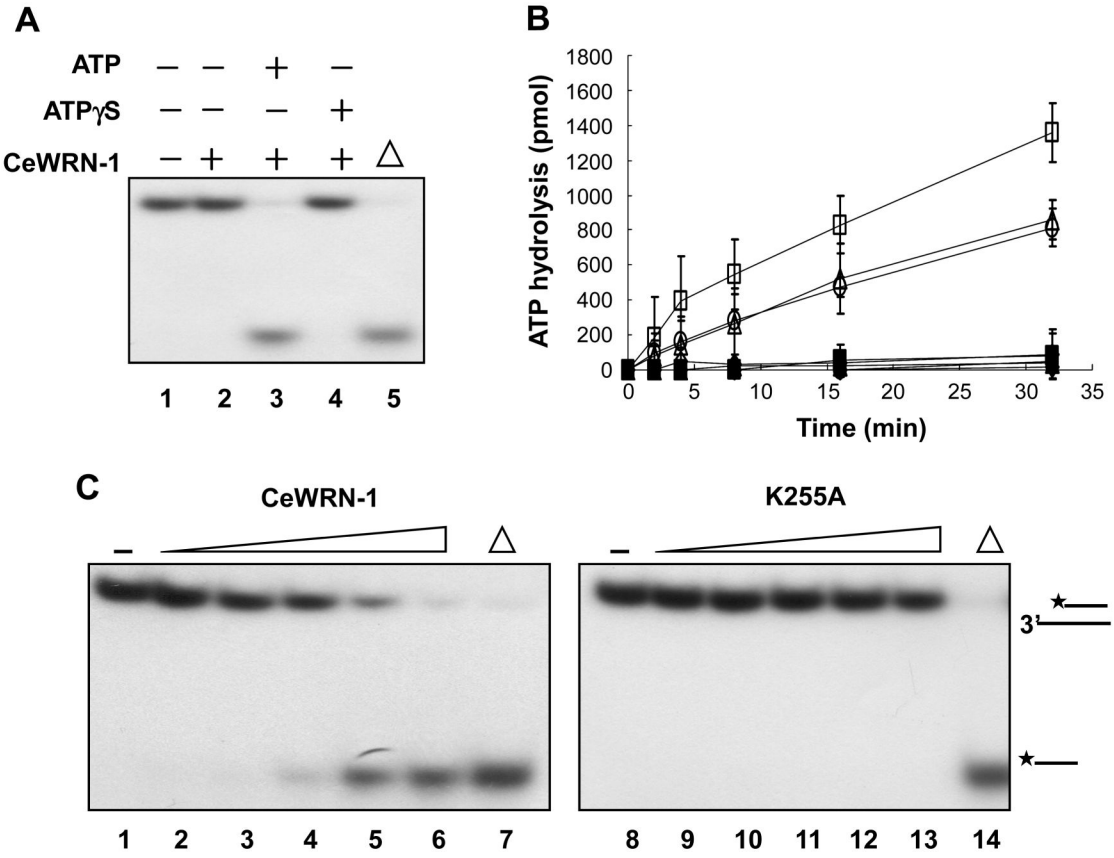



**Figure 3. Requirement for ATP hydrolysis and a conserved lysine residue in CeWRN-1 for helicase activity**

(A) Effect of ATP and ATP$\gamma$S on CeWRN-1 helicase activity. Reaction mixtures contained 1 nM substrate, 30 nM CeWRN-1, and 2 mM ATP (or 2 mM ATP$\gamma$S) as indicated. Helicase reactions were carried out as described in Materials and Methods. The reaction products were visualized by autoradiography: lane 1, no enzyme control; lane 2, no nucleotide control; lane 3, in the presence of ATP; lane 4, in the presence of ATP$\gamma$S; and lane 5, heat-denatured DNA substrate control. The substrate and product are shown at the right. (B) ATPase activity of CeWRN-1 in the presence of the indicated DNA effectors. Reaction mixtures contained 20 nM CeWRN-1 (white symbols) or 20 nM CeWRN-1-K255A (black symbols), 2 mM ATP, and 25 $\mu$g/mL DNA effector, and reactions were carried out at 37 °C for the indicated times. The amount of inorganic phosphate ($P_i$) released by ATP hydrolysis was determined as described in Materials and Methods: (△ and ▲) supercoiled plasmid, (○ and ●) blunt-ended linear dsDNA, (□ and ■) circular M13mp18 ssDNA, and (◇ and ◆) no DNA. (C) Comparative helicase assay for wild-type CeWRN-1 and CeWRN-1-K255A. Reactions were performed with 1 nM DNA substrate and varying amounts of purified wild-type CeWRN-1 (lanes 1–7) and mutant CeWRN-1-K255A (lanes 8–14) at 37 °C for 15 min. The reaction products were analyzed by 10% neutral PAGE. Radiolabeled DNAs were visualized by autoradiography: lanes 1 and 8, no enzyme control; and lanes 7 and 14, heat-denatured DNA substrate control. Lanes 2–6 show wild-type CeWRN-1 protein titration (2.2, 4.4, 8.8, 17.6, and 35.3 nM, respectively), and lanes 9–13 show CeWRN-1-K255A protein titration (3.1, 6.2, 12.5, 25, and 50 nM, respectively). The substrate and product are indicated schematically at the right.

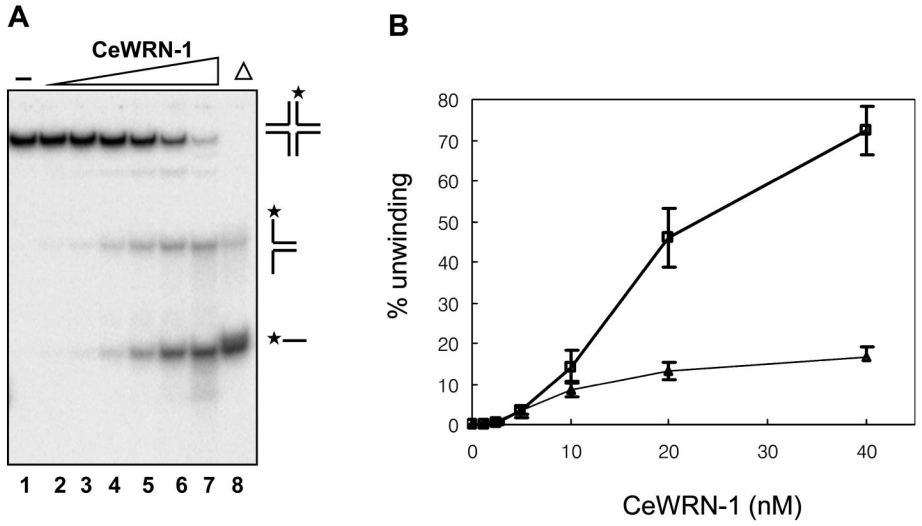



**Figure 4. CeWRN-1 helicase activity on a Holliday junction**

(A) Helicase reactions (10 $\mu$L) were performed by incubating varying amounts of CeWRN-1 with 1 nM synthetic Holliday junction at 37 °C for 15 min. The reaction products were analyzed via 10% nondenaturing PAGE and were subjected to phosphorimaging analysis: lane 1, no enzyme control; and lane 8, heat-denatured DNA substrate control. Lanes 2–7 show a protein titration (1.25, 2.5, 5, 10, 20, and 40 nM, respectively). The substrate and product are indicated schematically at the right. (B) Quantification of the data shown in panel A. The relative amount of dissociated product is expressed as the percentage of total DNA. The dissociated products include a forked duplex [the products of branch migration (△)] and ssDNA (□). Background levels of dissociated species have been subtracted. Data represent the means of at least three independent experiments ± SD.

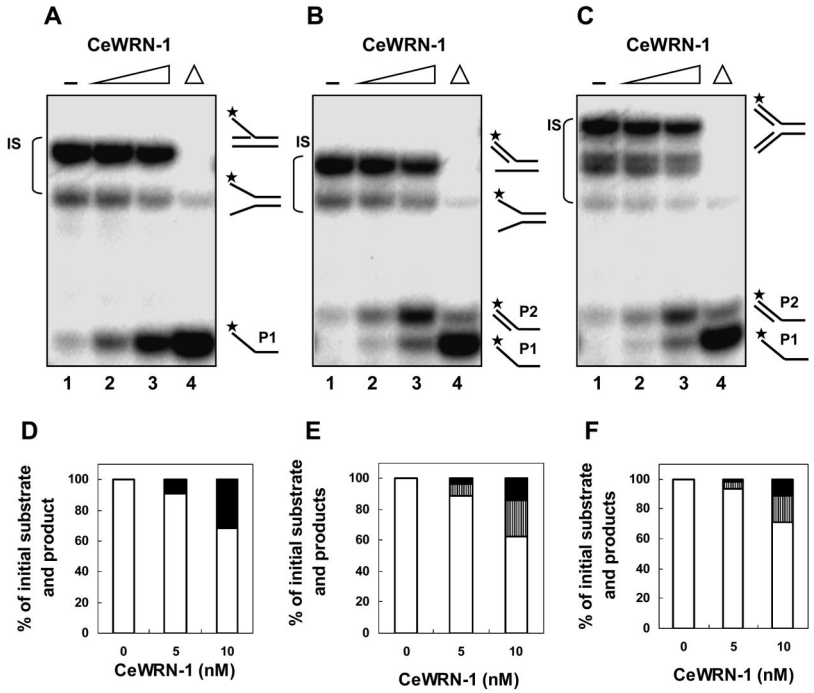



**Figure 5. Unwinding of flap DNA and three junction substrates by CeWRN-1**

The 5′ flap substrate (A), the 3′ flap (B), and the three-way junction (C) were incubated with varying concentrations of CeWRN-1 at 37 °C for 15 min. Reaction products were analyzed as described in Materials and Methods and were subjected to phosphorimaging analysis. Lane 1 contained no enzyme. Lane 4 contained heat-denatured DNA substrate. Lanes 2 and 3 show a protein titration (5 and 10 nM, respectively). The positions of the initial substrates (IS) and products (P1 or P2) are shown at the left or right. (D–F) Quantification of the data from panels A (D), B (E), and C (F). The percentages of IS and products were calculated using ImageQuant: (white bars) initial substrate, (black bars) single-stranded product (P1), and (gray bars) partial duplex product (P2).

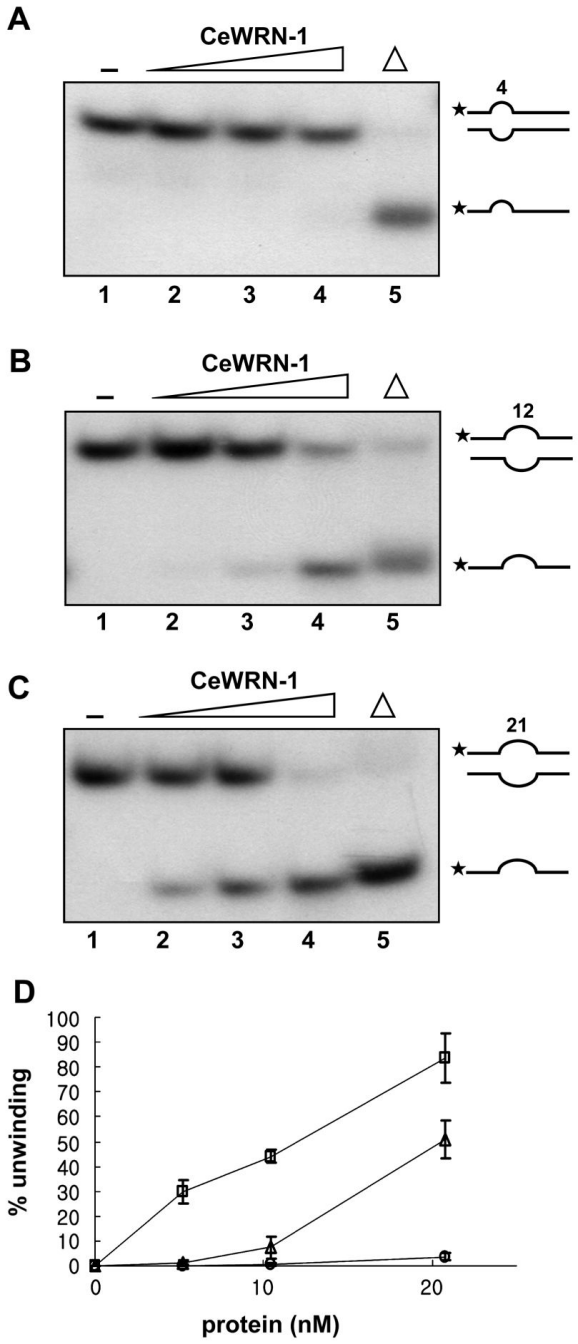



**Figure 6. Unwinding of bubble substrates by CeWRN-1**

Helicase reactions were performed by incubating the indicated concentrations of CeWRN-1 with 1 nM 4 bp bubble (A), 1 nM 12 bp bubble (B), or 1 nM 21 bp bubble (C) at 37 °C for 15 min: lane 1, no enzyme control; lane 5, heat-denatured DNA substrate control; and lanes 2–4, a protein titration (5.2, 10.4, and 20.8 nM, respectively). The reaction products were analyzed as described in Materials and Methods. The substrate and product are shown schematically at the right. (D) Quantitation of the data shown in panels A–C. The unwinding percentage is expressed as described in Materials and Methods. Data represent the means of at least three independent experiments ± SD: (○) 4 bp bubble, (△) 12 bp bubble, and (□) 21 bp bubble.

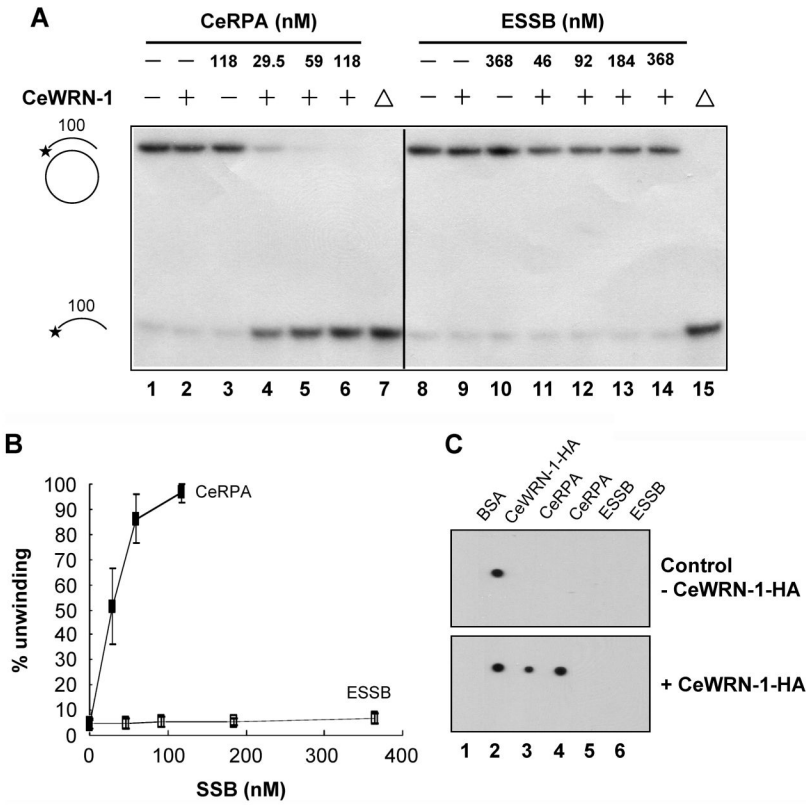
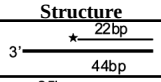
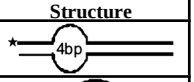
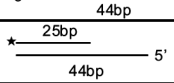
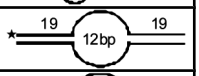
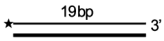
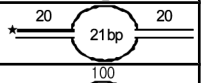
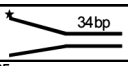
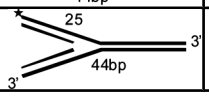



**Figure 7. Stimulation of CeWRN-1 helicase activity on a 100 bp partial DNA duplex substrate**

(A) CeWRN-1 protein (40 nM) was incubated with the 100 bp partial duplex in the presence of the indicated concentrations of CeRPA or ESSB protein under the standard helicase reaction conditions as described in Materials and Methods: lanes 1 and 8, no enzyme control; lanes 2 and 9, CeWRN-1 control; lane 3, CeRPA control; lane 10, ESSB protein control; lanes 7 and 15, heat-denatured DNA substrate control; lanes 4–6, a protein titration (CeRPA at 29.5, 59, and 118 nM, respectively); and lanes 11–14, a protein titration (ESSB at 46, 92, 184, and 368 nM, respectively). (B) Quantitation of results presented in panel A: (■) CeRPA and (□) ESSB protein. Percentage displacement is expressed as a function of SSB protein concentration. Data represent the means of at least three independent experiments ± SD. (C) Dot blot assay. BSA, CeWRN-1, CeRPA, and ESSB as indicated were immobilized on PVDF membranes. The membranes were blocked for 2 h at 4 °C and incubated in the absence or presence of CeWRN-1 as indicated at 4 °C overnight. After being washed, the membranes were incubated with monoclonal anti-HA antibody. Western blotting was performed as described in Materials and Methods.

**Table 1**

Structures of DNA Substrates for Helicase Assays

| Name | Structure | Name | Structure |
|---|---|---|---|
| 3′-overhang (1+2) |   | 4bp bubble (10+11) |   |
| 5′-overhang (2+3) |   | 12bp bubble (12+13) |   |
| Blunt-end duplex (4+5) |   | 21bp bubble (14+15) |   |
| forked duplex (6+7) |   | 100bp M13mp 18 Partial duplex |   |
| forked duplex (8+9) |   | FLAP (2+16+18) |   |
| FLAP (2+16+17) |   | Holliday-Junction (19+20+21+22) |   |
| Synthetic Replication fork (2+14+15+16) |   | | |

★Radioactive label at 5′