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Physical and Functional Interactions of *Caenorhabditis elegans* WRN-1 Helicase with RPA-1

Moonjung Hyun,[†,⊥] Sojin Park,[†] Eunsun Kim,[†] Do-Hyung Kim,[‡] Se-Jin Lee,[§] Hyeon-Sook Koo,[§] Yeon-Soo Seo,[∥] and Byungchan Ahn*[,†]

[†]Department of Life Sciences, University of Ulsan, Ulsan 680-749, Korea

[‡]Stowers Institute for Medical Research, 1000 East 50th Street, Kansas City, Missouri 64110, United States

[§]Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Korea

[∥]Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea

Supporting Information

ABSTRACT: The *Caenorhabditis elegans* Werner syndrome protein, WRN-1, a member of the RecQ helicase family, has a 3′−5′ DNA helicase activity. Worms with defective *wrn-1* exhibit premature aging phenotypes and an increased level of genome instability. In response to DNA damage, WRN-1 participates in the initial stages of checkpoint activation in concert with *C. elegans* replication protein A (RPA-1). WRN-1 helicase is stimulated by RPA-1 on long DNA duplex substrates. However, the mechanism by which RPA-1 stimulates DNA unwinding and the function of the WRN-1−RPA-1 interaction are not clearly understood. We have found that WRN-1 physically interacts with two RPA-1 subunits, CeRPA73 and CeRPA32; however, full-length WRN-1 helicase activity is stimulated by only the CeRPA73 subunit, while the WRN-$1_{162-1056}$ fragment that harbors the helicase activity requires both the CeRPA73 and CeRPA32 subunits for the stimulation. We also found that the CeRPA73_{1-464} fragment can stimulate WRN-1 helicase activity and that residues 335−464 of CeRPA73 are important for physical interaction with WRN-1. Because CeRPA73 and the CeRPA73_{1-464} fragment are able to bind single-stranded DNA (ssDNA), the stimulation of WRN-1 helicase by RPA-1 is most likely due to the ssDNA binding activity of CeRPA73 and the direct interaction of WRN-1 and CeRPA73.

RecQ homologues have been identified in *Escherichia coli*, yeast, *Caenorhabditis elegans*, mice, and humans.[1] The human RecQ proteins include RECQ1, BLM (Bloom), WRN (Werner), RTS (RECQ4), and RECQ5. Mutations to the BLM, WRN, and RTS genes lead to Bloom syndrome, Werner syndrome, and Rothmund-Thomson syndrome, respectively. These conditions are characterized by premature aging, predisposition to cancer, and developmental abnormalities.[2,3] Cells defective in the human RecQ genes exhibit genome instability, deficiencies in replication, and defects in DNA repair.[4]

RecQ proteins commonly have ATP-dependent 3′−5′ helicase activity and can act on key DNA replication and repair intermediates.[5−10] In addition to their catalytic properties, RecQ helicases interact with a number of key protein partners to participate in replication and repair functions.[2] Thus, RecQ helicases have been proposed to play roles in the maintenance of genomic stability through their catalytic activities and protein interactions.

Human WRN has 3′−5′ ATP-dependent helicase, 3′−5′ exonuclease, and ssDNA annealing activities.[8,11−15] WRN is active in resolving a variety of DNA substrates, including forks, flaps, displacement loops (D-loops), bubbles, Holliday junctions, and G-quadruplexes, all of which represent intermediates in the DNA replication and repair processes.[16] In particular, WRN can unwind long DNA duplexes in a manner dependent on replication protein A (RPA), a single-stranded DNA-binding protein.[17,18] In addition, WRN physically interacts with the RPA protein,[17] suggesting the potential involvement of WRN in DNA replication, repair, and recombination.

Four RecQ homologues have been identified in *C. elegans*: *recq1* (K02F3.12), *him-6* (T04A11.6), *wrn-1* (F18C5.2), and *recq5* (E03A3.2).[1,19−22] *C. elegans* with a mutation in *wrn-1* or treated with *wrn-1* RNAi manifests premature aging phenotypes and developmental defects, as shown using ionizing radiation (IR).[19] Our previous study demonstrated that *C. elegans* WRN-1 (WRN-1) has a 3′−5′ ATP-dependent helicase activity and that it unwinds various DNA structures.[23] Moreover, *C. elegans* RPA-1 stimulates WRN-1 helicase activity against a long DNA duplex substrate, suggesting that WRN-1 may interact with RPA-1 in vivo and function in DNA metabolic processes associated with RPA-1. A recent report has demonstrated that WRN-1 functions upstream of ATM/ATR in response to IR and hydroxyl urea (HU) and that WRN-1 can influence the formation of RPA-1 foci,[24] implying that WRN-1 may act as a checkpoint for DNA damage and replication blockade. The mechanism for stimulation of DNA unwinding by RPA-1

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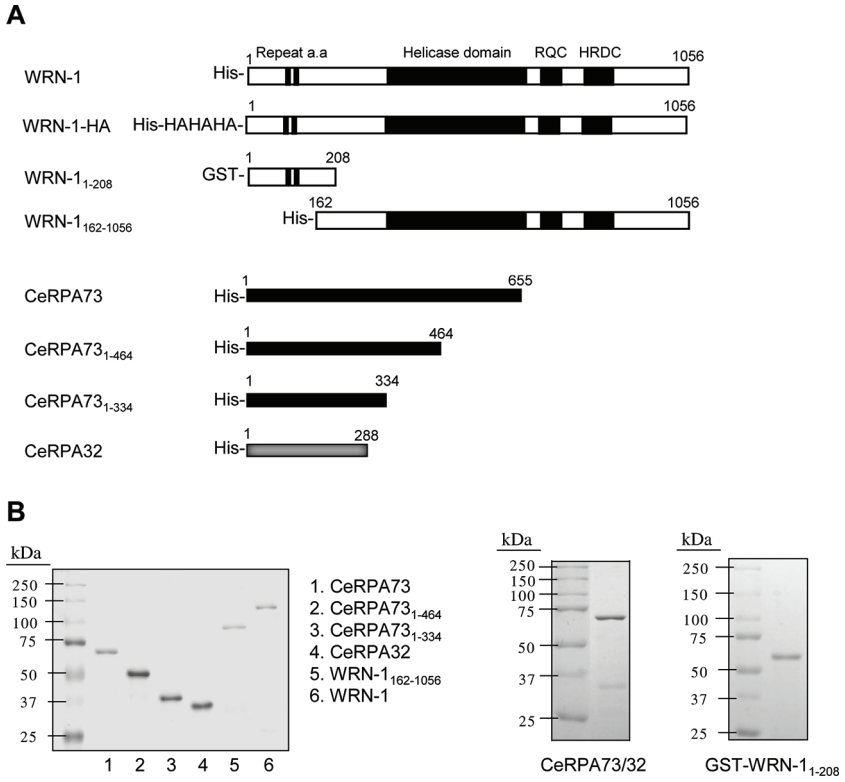

Figure 1. Recombinant WRN-1, the WRN-1 fragment, the RPA-1 and CeRPA subunits, and the CeRPA fragments. (A) Scheme of WRN-1 and RPA-1 proteins employed in this study. Full-length WRN-1, WRN-$_{1162-1056}$, the CeRPA73–CeRPA32 heterodimer, CeRPA73, CeRPA32, CeRPA73$_{1-334}$, and CeRPA73$_{1-464}$ were tagged with six histidines. CeWRN-1$_{1-208}$ was tagged with glutathione *S*-transferase. (B) SDS–PAGE analysis of purified proteins. Purified proteins were analyzed by 8% SDS–PAGE and stained with Coomassie Blue: lane 1, CeRPA73 (1.5 μg); lane 2, CeRPA73$_{1-464}$ (1.5 μg); lane 3, CeRPA73$_{1-334}$ (1.5 μg); lane 4, CeRPA32 (1.5 μg); lane 5, WRN-1$_{162-1056}$ (1 μg); lane 6, WRN-1 (1 μg).

has not yet been characterized. Furthermore, how the cellular function of WRN-1 is linked to RPA-1 remains unclear.

Because physical interactions between human RPA and the WRN or BLM helicases have been reported to be important for RPA-mediated stimulation of helicase-catalyzed DNA unwinding,[25−27] we characterized the interaction of WRN-1 with RPA-1 in vivo and in vitro. We identified residues 335–464 of CeRPA73 as being important for the physical interaction with WRN-1. In germline cells of *C. elegans* treated with camptothecin (CPT), WRN-1 localized to discrete foci along with RPA-1.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Recombinant DNA. *E. coli* BL21A1 [genotype *F*$^-$ *ompT hsdS*$_B$ (*r*$_B^-$*m*$_B^-$)*gal dcm araB::T7RNAP-tetA*] was used to express the recombinant proteins.

Polymerase chain reaction products for constructing WRN-1$_{162-1056}$, CeRPA73, CeRPA73$_{1-334}$, CeRPA73$_{1-464}$, and CeRPA32 were cloned into expression vector pDEST17 for His tagging (Gateway Cloning System, Invitrogen, Carlsbad, CA). The recombinant pDEST17 constructs were transformed into *E. coli* BL21A1 for expression.

Proteins. WRN-1, hemagglutinin (HA)-tagged WRN-1 (WRN-1-HA), WRN-1$_{162-1056}$, CeRPA73, CeRPA73$_{1-334}$, CeRPA73$_{1-464}$, and CeRPA32 were purified as previously described.[21] WRN-1$_{1-208}$ was purified as previously described.[19] The RPA-1 CeRPA73–CeRPA32 heterodimer was purified as previously described.[28] The quality of the purified proteins was determined by SDS–PAGE followed by Coomassie Blue staining (Figure 1B).

DNA Helicase Substrates. The PAGE-purified oligonucleotides used as substrates for the DNA helicase were synthesized by Integrated DNA Technologies (Coralville, IA). A single oligonucleotide at the 5′ end of the 22 and 34 bp forked duplex substrates was labeled with [γ-^{32}]ATP (3000 Ci/mmol) (Amersham Radiochemicals, Piscataway, NJ) using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) for 30 min at 37 °C and heat-inactivated for 10 min at 95 °C. Labeled oligonucleotides were annealed to unlabeled complementary strands at a 1:2 (or 1:4) molar ratio by incubation at 90 °C for 5 min followed by slow cooling to room temperature. The 100-mer M13mp18 partial duplex substrate was constructed using an oligonucleotide complementary to positions 6039–6138 of the M13mp18 ssDNA circular DNA (New England Biolabs).

DNA Helicase Assay. Proteins and radiolabeled DNA substrates (1 nM) were incubated in 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 bovine serum albumin (BSA)] in a final volume of 10 μL. Reaction mixtures were incubated at 37 °C for 15 min and reactions 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). The helicase reaction products were analyzed on nondenaturing 10% (19:1 acrylamide:bisacrylamide) (Bio-Rad, Hercules, CA) polyacrylamide gels. Radiolabeled DNA species were visualized using Kodak Biomax MR Film (GE Life Science, Piscataway, NJ) and quantitated using Scion Image (NIH Image).

The percent of unwinding was calculated from the relationship % unwinding = 100[P/(S + P)], where P is the

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amount of product and *S* is the amount of substrate. The values of *P* and *S* were corrected after subtracting the background values obtained in the no-protein and heat-denatured substrate controls.

Antibody Preparation. WRN-1 and CeRPA73 polyclonal antibodies were produced using the antibody production service of AbFRONTIER (Seoul, Korea). Rabbit anti-WRN-1 and mouse anti-CeRPA73 polyclonal antibodies were used for immunohistochemistry and Western blot analyses. The Alexa Fluor-488-conjugated goat anti-rabbit and Alexa Fluor-594-conjugated goat anti-mouse secondary antibodies were obtained from Invitrogen.

Immunoprecipitation. The WRN-1-HA, CeRPA73-His, and CeRPA32-His proteins were incubated with protein G agarose (Santa Cruz Biotechnology, Santa Cruz, CA). Precleared CeRPA73-His (10 pmol) or CeRPA32-His (10 pmol) was incubated with WRN-1-HA protein (10 pmol) in 100 μL of reaction buffer [50 mM Tris (pH 8.0), 120 mM NaCl, and 0.5% NP-40] at 4 °C for 4 h. The supernatant was incubated with 1.0 μL of mouse anti-HA (Sigma-Aldrich, St. Louis, MO) or 10 μL of monoclonal anti-HA agarose at 4 °C for 16 h. The protein−antibody complex was precipitated with 10 μL of protein G agarose at 4 °C for 2 h. Precipitates were washed with reaction buffer, and proteins were resolved by SDS−PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were immunoblotted with a monoclonal anti-His antibody (Sigma-Aldrich), and the WRN-1 and CeRPA subunits were detected using an ECL kit (Amersham).

C. elegans extracts were prepared from whole worms that were washed from NGM plates using 1× PBST (1× PBS with 0.1% Tween 20). Pellets were rinsed with 1× PBST, harvested in a clinical centrifuge, frozen in liquid nitrogen, and ground. The ground pellets were resuspended in lysis buffer [50 mM Tris (pH 8.0), 120 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100, 10% glycerol, 1 mM PMSF, and a protease inhibitor cocktail tablet (Roche, Mannheim, Germany)] and lysed by sonication (10 bursts at 10 s intervals). The whole cell extract was precleared with protein G agarose beads (10 μL) for 2 h at 4 °C and incubated with either a rabbit anti-WRN-1 (1:100) or a rabbit anti-IgG antibody (negative control) (1:100) for 16 h at 4 °C in reaction buffer. After incubation, 10 μL of protein G agarose was added to each sample and incubated for 2 h at 4 °C. Beads were pelleted (5000*g* for 5 min at 4 °C) and washed four times with 500 μL of reaction buffer. Bound proteins were eluted by being boiled in protein sample buffer and analyzed by SDS−PAGE and Western blotting as described above.

Immunohistochemistry. Worms were grown to L4 stage larvae at 20 °C. L4 stage worms were transferred to a new NGM plate containing 50 μM CPT. Twenty-four hours after the worms had been treated with 50 μM CPT, the gonads from the worms were extruded by cutting the heads from hermaphrodite adult worms with a fine-gauge needle and were fixed in 4% paraformaldehyde (PFA). PFA was removed, and the dissected gonads were washed three times with a 1× PBS/glycine mixture and permeabilized in PBSBT (1× PBS, 0.5% BSA, and 0.1% Triton X-100) for 10 min. Three washes with PBST (1× PBS and 0.1% Tween 20) were performed, and gonads were blocked with PBSBT containing 2% nonfat milk at 4 °C overnight. Primary antibodies were diluted in PBSBT with 2% nonfat milk (1:500 for WRN-1 and RPA-1) and incubated in a humid chamber for 12 h at 4 °C. Gonads were washed three times for 10 min each with PBST before being incubated with secondary antibodies (Alexa Fluor-488-conjugated goat anti-rabbit and Alexa Fluor-594-conjugated goat anti-mouse secondary antibodies from Molecular Probes, Carlsbad, CA) for 1 h at room temperature in a dark chamber. After being stained with 4,6-diamidino-2-phenylindole (DAPI) (1 mg/mL), worms were mounted on agarose pads and inspected using a Carl Zeiss microscope equipped with epifluorescence filters.

RESULTS

Functional Interaction of WRN-1 with the CeRPA73 Subunit. We have previously demonstrated that a CeRPA73−CeRPA32 heterodimer allows WRN-1 helicase to efficiently unwind partial duplex DNA consisting of a 100-mer hybridized to ssM13 DNA.[23] This stimulation is specific for RPA-1 because *E. coli* SSB failed to stimulate.[23]

To identity the subunit(s) responsible for stimulating WRN-1 helicase activity, we measured the extent of WRN-1-catalyzed unwinding of a 100-mer partial DNA duplex in the presence of each CeRPA subunit. We purified recombinant WRN-1, the CeRPA73−CeRPA32 heterodimer, the CeRPA73 subunit, and the CeRPA32 subunit from *E. coli* (Figure 1A,B). WRN-1 was incubated with a 100-mer partial duplex in the presence of the indicated concentrations of the CeRPA73−CeRPA32 heterodimer, CeRPA73, or CeRPA32, and the reaction products were analyzed on native 10% polyacrylamide gels (Figure 2)

The substrate was unwound by full-length WRN-1 in the presence of the CeRPA73 subunit, but not in the presence of CeRPA32 (Figure 2A,B). More substrate was unwound by WRN-1 in the presence of the CeRPA73−CeRPA32 heterodimer than in the presence of only CeRPA73 (Figure 2B). These results suggest that the CeRPA73 subunit is responsible for stimulating WRN-1 helicase, and both subunits are likely needed for efficient unwinding.

Physical interactions between human RPA and the WRN or BLM helicases have been reported to play important roles in the mechanism by which RPA stimulates helicase-catalyzed DNA unwinding.[25−27] Thus, we determined whether the CeRPA73−CeRPA32 heterodimer physically interacts with WRN-1 using immunoprecipitation (Figure 3). Either CeRPA73 or CeRPA32 was incubated with purified recombinant WRN-1-HA [hemagglutinin-tagged WRN-1 (Figure 1A)] to detect in vitro interactions. Incubated samples were bound to anti-HA agarose beads, and the bound proteins were analyzed by Western blotting. CeRPA73 and CeRPA32 were identified with an anti-His tag antibody (Figure 3A, lanes 4−6), suggesting that WRN-1 interacted with either CeRPA73 or CeRPA32. No cross reactivity of the anti-HA antibody against CeRPA73 and CeRPA32 was detected (Figure S1A of the Supporting Information).

Next, we ascertained whether similar interactions occurred in vivo using *C. elegans* extracts from either wild-type N2 or *wrn-1(gk99)* mutants. Precleared extracts were incubated with polyclonal antibodies against either WRN-1 or CeRPA73 and analyzed by Western blotting for WRN-1 or CeRPA. When N2 extracts were incubated with anti-WRN-1, WRN-1 and CeRPA73 were detected (Figure 3B). CeRPA73 and WRN-1 were also detected after immunoprecipitation of N2 extracts with anti-CeRPA73 (Figure 3B). In contrast, WRN-1 was not detected in *wrn-1* mutant extracts (Figure 3B). Consistent with this observation, CeRPA73 was not detected in anti-WRN-1 immunoprecipitation of *wrn-1* mutant extracts (data not shown). Under the control conditions, WRN-1 and CeRPA73 were not detected upon immunoprecipitation with protein G or mouse IgG agarose beads (Figure 3B). This interaction was not

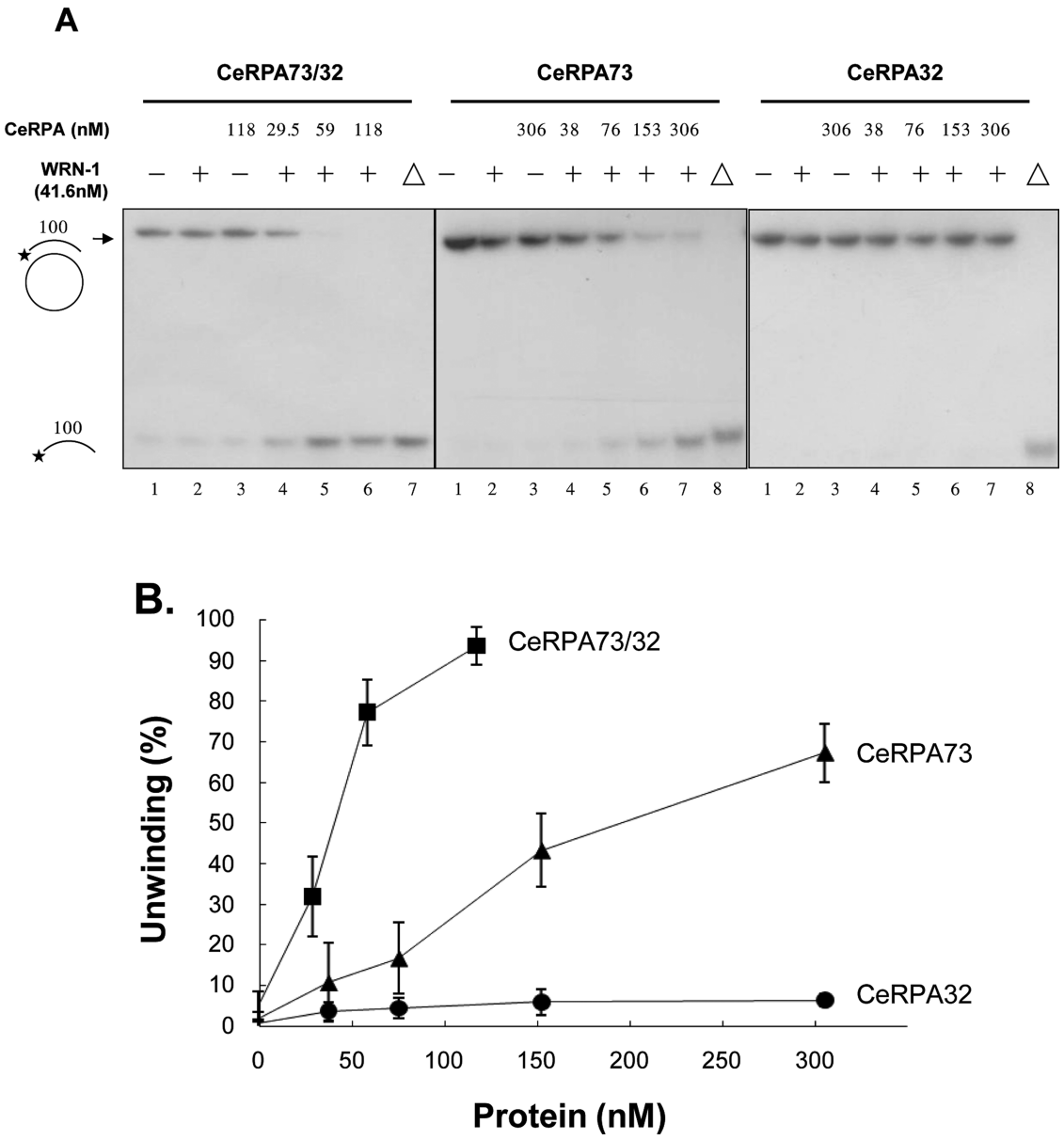

Figure 2. CeRPA73 subunit stimulates WRN-1 helicase activity on a 100-mer M13 partial duplex DNA. (A) WRN-1 protein (41.6 nM) was incubated with a 100-mer partial duplex in the presence of the indicated concentrations of the CeRPA73−CeRPA32 heterodimer, CeRPA73, or CeRPA32 under the standard helicase reaction conditions (see Experimental Procedures). The helicase reaction products were analyzed on native 10% polyacrylamide gels: lane 1, no enzyme control; lane 2, CeWRN-1 control; lane 3, CeRPA73−CeRPA32 heterodimer, CeRPA73 protein, and CeRPA32 protein controls; lanes 4−6, RPA-1 titration (CeRPA73−CeRPA32 at 29.5, 59, and 118 nM, respectively); lanes 4−7, titration of the CeRPA subunits (CeRPA73 at 38, 76, 153, and 306 nM and CeRPA32 at 38, 76, 153, and 306 nM, respectively). The heat-denatured DNA substrate control is indicated by open triangles. (B) Percent displacement of the radiolabeled DNA strand from the partial duplex substrate plotted vs RPA-1 concentration obtained in panel A: CeRPA73−CeRPA32 heterodimer (■), CeRPA73 (▲), and CeRPA32 (●). The helicase data are presented as the means of at least three independent experiments with the SD indicated by error bars.

mediated by DNA because the binding was not affected by the presence of ethidium bromide, demonstrating that WRN-1 and CeRPA interact directly (Figure S1B of the Supporting Information). These results suggest that WRN-1 interacts with two CeRPA subunits, but only CeRPA73 stimulates the DNA unwinding activity of WRN-1.

Physical and Functional Interaction of WRN-1 with CeRPA73$_{1-464}$. We used two C-terminal truncation mutants of CeRPA73 to map the CeRPA73 domains that physically interact with WRN-1. CeRPA73$_{1-334}$ and CeRPA73$_{1-464}$ were expressed as His-CeRPA fusion proteins, purified (Figure 1), and immunoprecipitated with anti-WRN-1. Bound protein samples were analyzed by Western blotting for the bound CeRPA73 fragments (Figure 4A).

CeRPA73$_{1-464}$ was detected with a monoclonal anti-HIS antibody in samples eluted from the WRN-1 immunoprecipitation (Figure 4A). WRN-1 was not detected in samples eluted after immunoprecipitation of CeRPA73$_{1-334}$ and WRN-1 (Figure 4A). These data indicate that residues 334−464 in the CeRPA73 subunit are involved in the physical interaction with WRN-1.

To improve our understanding of the importance of the physical interaction between WRN-1 and CeRPA73$_{1-464}$, we tested WRN-1 in a functional helicase assay against a forked

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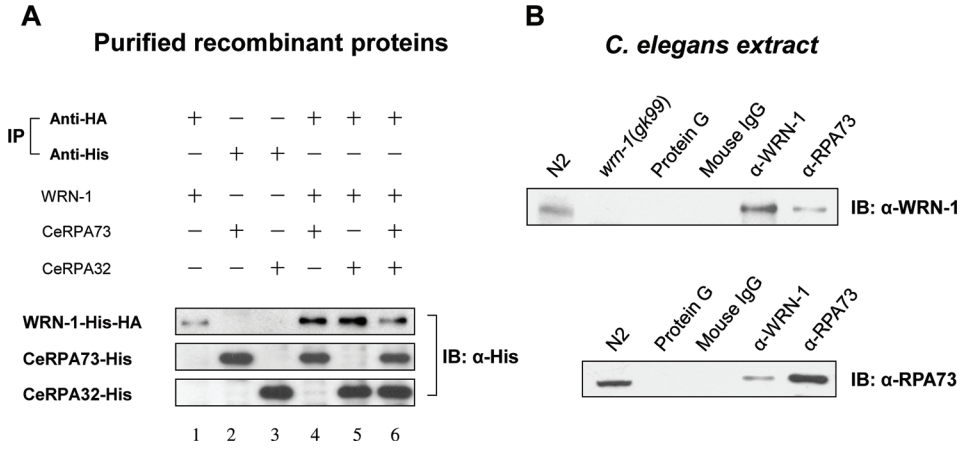

Figure 3. Physical interaction between WRN-1 and CeRPA in vitro and in vivo. (A) Purified recombinant CeRPA73 and CeRPA32 proteins were incubated with purified WRN-1-HA and immunoprecipitated with anti-HA agarose beads. The bound proteins were resolved on 7% polyacrylamide−SDS gels. Proteins were detected by Western blotting using an antibody against the six-His tag. (B) Precleared *C. elegans* N2 or *wrn-1(gk99)* extract was incubated with anti-CeRPA or anti-CeWRN-1 antibodies. After incubation, the protein and antibody mixtures were bound to protein G agarose beads. Co-immunoprecipitation was detected by Western blotting using anti-WRN-1 or anti-CeRPA73 antibodies.

duplex substrate in the presence of CeRPA73$_{1-464}$ (Figure 4B,C). The level of unwinding of the forked duplex increased with increasing amounts of CeRPA73$_{1-464}$, but CeRPA73$_{1-334}$ failed to stimulate (Figure S2A of the Supporting Information). These results suggest that the physical interaction is related to the functional interaction.

Physical and Functional Interaction of WRN-1$_{162-1056}$ with the CeRPA73−CeRPA32 Heterodimer. We used N-terminal and C-terminal truncation mutants to map the WRN-1 domains that physically interact with the CeRPA subunits. The N-terminal deletion mutant, WRN-1$_{162-1056}$, contains a helicase domain, and the C-terminal deletion mutant, WRN-1$_{1-208}$, contains acidic repeats (Figure 1). We tested the DNA unwinding activity of the purified WRN-1 fragments (Figure 1) using 22 and 34 bp forked duplex substrates. WRN-1$_{162-1056}$ unwound both forked duplexes, but the 34 bp duplex was unwound less than the 22 bp duplex (Figure S4A,B of the Supporting Information). No detectable unwinding by WRN-1$_{1-208}$ was observed, as expected (data not shown). However, full-length WRN-1 displaced the 34 bp duplex more than the WRN-1$_{162-1056}$ fragment (Figure S4A,B of the Supporting Information). These results suggest that the N-terminal region of WRN-1 is likely to be important for unwinding longer forked duplexes.

We used the two different WRN-1 fragments to determine which interacts with the CeRPA subunits by immunoprecipitation. WRN-1$_{162-1056}$ was incubated with CeRPA73 or CeRPA32 followed by immunoprecipitation with an anti-WRN-1 antibody. Bound proteins were detected by Western blotting using a monoclonal anti-His antibody (Figure 5A) to detect the RPA-1 subunits. The presence of CeRPA73 and CeRPA32 in the eluted samples indicated co-immunoprecipitation of both RPA-1 subunits with WRN-1$_{162-1056}$. No immunoprecipitation of the RPA-1 subunits was observed in the absence of WRN-1$_{162-1056}$. Moreover, no interaction between WRN-1$_{1-208}$ and CeRPA73 or CeRPA32 was detected via dot blot analysis (data not shown). These results suggest that WRN-1$_{162-1056}$ physically interacts with CeRPA73 and/or CeRPA32.

To understand the functional importance of the WRN-1−CeRPA interaction, we measured WRN-1$_{162-1056}$-catalyzed unwinding of a 34 bp forked duplex and a 100-mer partial DNA duplex in the presence of the RPA-1 subunits. The degree of unwinding of the 34 bp forked duplex by WRN-1$_{162-1056}$ increased with increasing concentrations of the CeRPA73−CeRPA32 heterodimer (Figure 5B,C, lanes 4−6). In the absence of the heterodimer, WRN-1$_{162-1056}$ (42 nM) unwound approximately 25% of the 34 bp forked duplex substrate (Figure 5B, lane 3), and no unwinding was detected with the CeRPA73−CeRPA32 heterodimer (118 nM) (Figure 5B, lane 2). In contrast, the CeRPA73 subunit alone failed to detectably stimulate the DNA unwinding activity of WRN-1$_{162-1056}$ (lanes 11−13 in Figure 5B). With a 100-mer partial duplex substrate, WRN-1$_{162-1056}$ (128 nM) unwound up to 65% when exposed to increasing concentrations of the CeRPA73−CeRPA32 heterodimer (Figure 5D,E), although the extent of unwinding was less than with full-length WRN-1 (compare with Figure 2B). These results suggest that the WRN-1$_{162-1056}$ region physically and functionally interacts with the CeRPA subunits and that CeRPA32 may be involved in stimulating WRN-1$_{162-1056}$ helicase activity by CeRPA73 in the absence of the N-terminus of WRN-1.

Colocalization of WRN-1 and CeRPA in Response to CPT Treatment. The physical and functional interactions of WRN-1 with the CeRPA subunits and the stimulation of WRN-1 helicase activity by the CeRPA subunits suggest that WRN-1 may interact with the CeRPA subunits in vivo.

In mammalian cells, the overlap between immunostained WRN foci and RPA foci in response to genotoxic stresses indicates in vivo interactions in DNA damage processing.[29,30] Therefore, we examined the nuclear localization of WRN-1 and CeRPA73 in response to a DNA-damaging agent by immunostaining.

In untreated *C. elegans*, WRN-1 showed uniformly diffuse staining in the nucleoplasm of germline cells; however, WRN-1 was localized to foci in CPT-treated germline cells (Figure 6). Immunostaining with the anti-CeRPA73 antibody showed discrete CeRPA73-containing foci in response to CPT treatment, while CeRPA73 was evenly distributed throughout the nuclei in untreated germline cells. Intriguingly, the WRN-1 nuclear spots significantly colocalized with CeRPA73 foci. These results suggest that WRN-1 and RPA-1 might act cooperatively following CPT treatment. However, CeRPA73 was also detected in *wrn-1(gk99)* germ cells (manuscript in preparation), suggesting that WRN-1 is not essential for the formation of CeRPA73 foci in response to CPT.

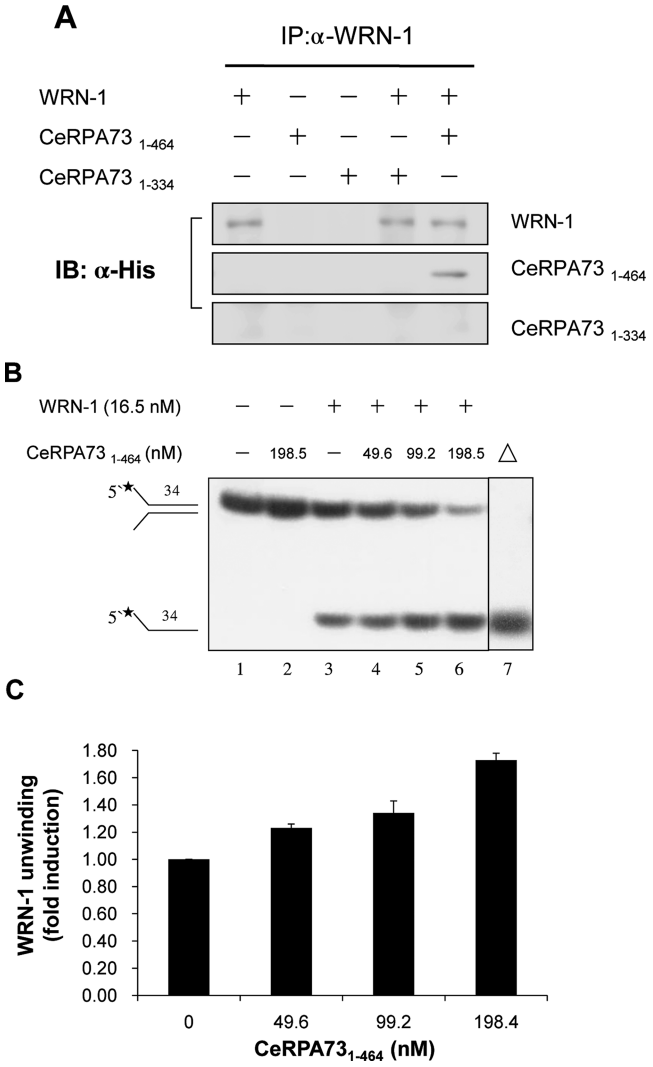

Figure 4. Mapping WRN-1 binding domains on CeRPA73. (A) Immunoprecipitation of $CeRPA73_{1-464}$ with WRN-1. The purified recombinant $CeRPA73_{1-464}$ fragment was incubated with WRN-1. After immunoprecipitation, the bound proteins were resolved on 7% polyacrylamide–SDS gels. Bound $CeRPA73_{1-464}$ was detected by Western blotting using an anti-His antibody. (B) Stimulation of CeWRN-1 by $CeRPA73_{1-464}$. A 34 bp forked duplex (34 bp duplex region) and WRN-1 were incubated with increasing amounts of $CeRPA73_{1-464}$ under the standard helicase reaction conditions (see Experimental Procedures). The helicase reaction products were analyzed on native 10% polyacrylamide gels: lane 1, no enzyme control; lane 2, 198.5 nM $CeRPA73_{1-464}$; lane 3, 16.5 nM WRN-1; lanes 4–6, a $CeRPA73_{1-464}$ titration (49.6, 99.2, and 198.5 nM, respectively). A heat-denatured DNA substrate control is indicated by the open triangles. (C) Quantitation of the results depicted in panel B. Increased levels of WRN-1 unwinding relative to lane 3 (in the absence of $CeRPA73_{1-464}$) are presented at indicated $CeRPA73_{1-464}$ concentrations. Data are the means of at least three independent experiments with the SD indicated by error bars.

DISCUSSION

The enzymatic activity of WRN-1 and its mutant phenotypes has been characterized, but the cellular functions of WRN-1 are not fully understood. Our previous finding that WRN-1 helicase activity is stimulated by the CeRPA73–CeRPA32 heterodimer suggested that WRN-1 has a low processivity for unwinding duplex DNA and that WRN-1 may act cooperatively with RPA-1 in DNA metabolic processes.

Unwinding processivity may be due to the ability to keep the displaced strand (the 3′ tail) away from the complementary strand (the 5′ tail), preventing the two strands from re-annealing. WRN-1 may hold the displaced strand weakly, resulting in low processivity. We found that the CeRPA73 subunit enhances WRN-1 helicase activity during unwinding of a 100-mer partial duplex substrate and that the CeRPA73 subunit physically interacts with WRN-1 in vitro and in vivo. In addition, CeRPA73 has an ssDNA binding activity (Figure S3 of the Supporting Information). Thus, it is suggested that CeRPA73 may provide further stabilization of binding of WRN-1 to the displaced strand through CeRPA73 ssDNA binding activity and a physical interaction with WRN-1.

Similar specific RPA stimulation was also found for the WRN and BLM helicases.[25−27] In addition, the *E. coli* RecQ helicase activity was stimulated by *E. Coli* SSB.[31] Hence, the stimulation of RecQ helicase activity by ssDNA binding proteins may be evolutionarily conserved.

However, CeRPA stimulation of WRN-1 helicase-catalyzed DNA unwinding in *C. elegans* is slightly different from that in humans. The human hRPA70 subunit stimulated the helicase activity of WRN to the same extent as the hRPA heterotrimer, but the CeRPA73–CeRPA32 heterodimer stimulates WRN-1 helicase activity more than CeRPA73. WRN physically interacted only with the hRPA70 subunit, but WRN-1 interacts with both the CeRPA73 and CeRPA32 subunits with different binding affinities. CeRPA73 exhibited a higher affinity for WRN-1 than CeRPA32, but the affinity of WRN-1 for the CeRPA73–CeRPA32 heterodimer was much higher than that of each subunit (Figure 5S), suggesting a synergistic effect of each subunit on WRN-1 interaction. Because CeRPA32 was not able to bind to ssDNA (Figure 3S), CeRPA32 may act as an accessory factor for formation of a more stable WRN-1–CeRPA73–CeRPA32 complex through its physical interaction with both CeRPA73 and WRN-1.

The acidic direct repeat domain of WRN mediates the physical interaction between WRN and the hRPA70 subunit.[26] The region of the *Xenopus* homologue of WRN, FFA-1, that is required for interaction with RPA contains a single acidic repeat element.[32,33] Because WRN-1 contains two putative acidic direct repeat domains in the N-terminal region,[21] we generated the following two WRN-1 fragments: $WRN\text{-}1_{1-208}$ harboring the acidic direct repeats and $WRN\text{-}1_{162-1056}$ containing the helicase domain. The interaction of $WRN\text{-}1_{162-1056}$ with CeRPA73 and CeRPA32 was detected by in vitro immunoprecipitation, but $WRN\text{-}1_{1-208}$ did not interact. Thus, the region of WRN-1 that interacts with RPA-1 seems to be different from other WRN homologues. In humans, the N-terminal region (residues 239–499) of WRN containing the acidic direct repeats mediates the interaction with RPA70.[26]

Purified $WRN\text{-}1_{162-1056}$ had helicase activity that was more efficient in unwinding a short forked duplex (22 bp) than a long forked duplex (34 bp), and it poorly unwound a 100-mer partial duplex substrate. Compared to full-length WRN-1 against the 34 bp forked duplex, the helicase activity of $WRN\text{-}1_{162-1056}$ against forked duplexes was significantly reduced (Figure S4A,B of the Supporting Information). Thus, the deleted N-terminal region of WRN-1 may be involved in efficient unwinding of a long-forked substrate through an induced conformational change that may cause WRN-1 to bind stably to the displaced strand.

Because purified $WRN\text{-}1_{162-1056}$ physically interacted with CeRPA73 and CeRPA32, we analyzed the functional

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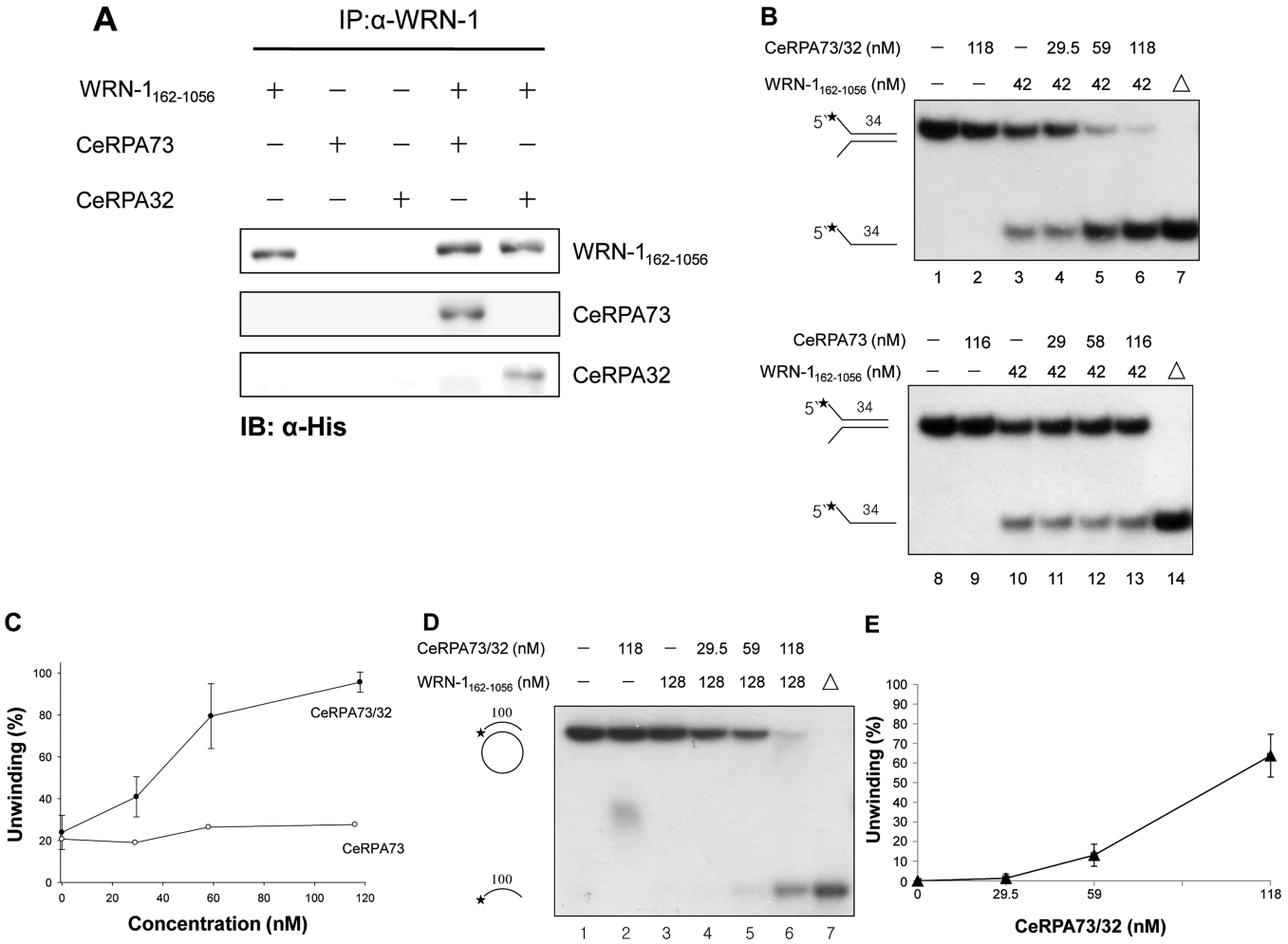

Figure 5. WRN-$1_{162-1056}$ functionally and physically interacts with CeRPA subunits. (A) Physical interaction of WRN-$1_{162-1056}$ with CeRPA73 or CeRPA32. The purified recombinant WRN-$1_{162-1056}$ fragment and CeRPA73 or CeRPA32 protein were immunoprecipitated with anti-WRN-1 and detected by Western blotting using an anti-His antibody. (B) Helicase reaction of WRN-$1_{162-1056}$ in the presence of the CeRPA73−CeRPA32 heterodimer and CeRPA73. The forked duplex (the 34 bp duplex region) and WRN-$1_{162-1056}$ were incubated with increasing amounts of the CeRPA73−CeRPA32 heterodimer or CeRPA73 under standard helicase reaction conditions (see Experimental Procedures). The helicase reaction products were analyzed on native 10% polyacrylamide gels: lane 1, no enzyme control; lane 2, 118 nM CeRPA73−CeRPA32 heterodimer; lane 3, 42 nM WRN-$1_{162-1056}$; lanes 4−6, a CeRPA73−CeRPA32 heterodimer titration (29.5, 59, and 118 nM, respectively); lane 8, no enzyme control; lane 9, 115 nM CeRPA73; lane 10, 42 nM WRN-$1_{162-1056}$; lanes 11−13, a CeRPA73 titration (29, 58, and 116 nM, respectively). The heat-denatured DNA substrate control is indicated by the open triangles. (C) Percent displacement of the radiolabeled DNA strand from the substrates plotted vs protein concentration of the CeRPA heterodimer and CeRPA73 depicted in panel B. The helicase data are presented as the means of at least three independent experiments with the SD indicated by error bars. (D) WRN-$1_{162-1056}$ (128 nM) was incubated with a 100 bp partial duplex with the indicated amounts of the CeRPA73−CeRPA32 heterodimer under standard helicase reaction conditions (see Experimental Procedures). The reaction products were analyzed on native 10% polyacrylamide gels: lane 1, no enzyme control; lane 2, CeRPA73−CeRPA32 heterodimer control; lane 3, WRN-$1_{162-1056}$ control; lanes 4−6, protein titration of the CeRPA73−CeRPA32 heterodimer (29.5, 59, and 118 nM, respectively); lane 7, heat-denatured DNA substrate control. (E) Quantitation of results in panel D: WRN-1 (■) and WRN-$1_{162-1056}$ (▲). The percentage of displacement is expressed as a function of the CeRPA73−CeRPA32 heterodimer concentration. Data are the means of at least three independent experiments with the SD indicated by error bars.

interaction between the CeRPA subunits and WRN-$1_{162-1056}$. CeRPA73 alone failed to stimulate unwinding of the 34 bp forked duplex (Figure 5B,C). Because full-length WRN-1 was stimulated by CeRPA73 alone, CeRPA73 may be involved in maintaining the changed conformation of WRN-1 by the N-terminal domain. However, the presence of both CeRPA73 and CeRPA32 stimulated unwinding of the 34 bp forked duplex (Figure 5B,C) and the 100-mer partial duplex substrates by WRN-$1_{162-1056}$ (Figure 5D,E). These results suggest that CeRPA32 may substitute the requirement of the N-terminal domain of WRN-1 and act as an accessory factor for the formation of a more stable complex between WRN-1 and CeRPA73 through a physical interaction with both proteins because CeRPA32 was not able to bind ssDNA (Figure S3 of the Supporting Information). This suggestion is further supported by the results depicted in Figure 2A where more unwinding occurred in the presence of both CeRPA73 and CeRPA32.

Previous studies have mapped the hRPA70 domain that interacts with WRN and thus revealed that the N-terminal half of hRPA70 mediates the physical interaction with WRN. These studies have indicated that the functional WRN−RPA interaction is mediated by residues 169−441 of hRPA70.[27] Mutants with C-terminal deletions of 175 (hRPA70ΔC442) or

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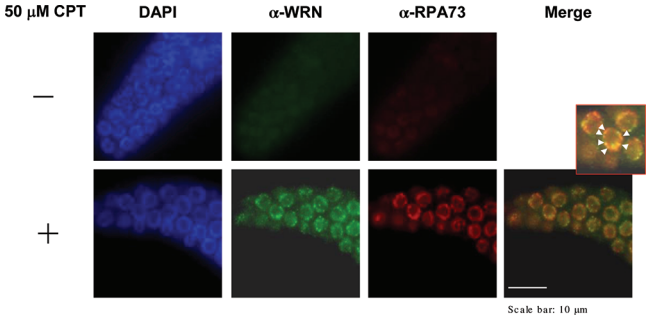

Figure 6. Colocalization of WRN-1 and RPA-1 in response to CPT in mitotic germ cells from *C. elegans*. Wild-type N2 worms from 1-day-old young adults were cultured for 6 h with CPT (50 μM). Mitotic germ cells were stained with antibodies against WRN-1 and RPA-1. Stained germline cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Magnification bars are 10 μm.

290 (hRPA70ΔC327) residues bound to WRN as tightly as wild-type hRPA, but the mutant hRPA70ΔC442 stimulated activity only to the same extent as wild-type hRPA.

Functional differences between the two hRPA70 fragments and the sequence alignment between CeRPA73 and hRPA70 led us to design two CeRPA73 proteins with C-terminal deletions ($CeRPA73_{1-464}$ and $CeRPA73_{1-334}$). The purified CeRPA73 fragments were tested for physical and functional interactions with WRN-1. $CeRPA73_{1-464}$ physically interacted with WRN-1 and stimulated its DNA unwinding activity (Figure 4A–C); however, $CeRPA73_{1-334}$ failed to physically and functionally interact with WRN-1 (Figure S2A of the Supporting Information) and to bind ssDNA, whereas $CeRPA73_{1-464}$ was able to bind ssDNA (Figure S3 of the Supporting Information). Thus, the ssDNA binding activity of CeRPA73 and the physical interaction with WRN-1 may be a molecular basis for the stimulation of WRN-1 helicase activity.

When *C. elegans* was treated with CPT, WRN-1 foci coincided with CeRPA73 foci. This colocalization pattern suggests that the two proteins function cooperatively in response to DNA damage induced by CPT. When DNA replication forks encounter CPT-induced nicks, replication fork arrest and DSBs result.[34−36] The CPT-induced DSBs are repaired by homologous recombination.[37−39] RPA is indispensable for DSB repair and homologous recombination. Thus, the colocalization of WRN-1 and RPA-1 indicates that the two proteins may act in concert to repair CPT-induced DSBs. Because the formation of WRN-1 foci in response to CPT treatment depends on CeRPA-1 (manuscript in preparation), CeRPA-1 may recognize DSBs before WRN-1 acts.

Possible roles of WRN-1 can be postulated on the basis of the biochemical properties of WRN-1 and the functions of WRN and RecQ helicases in other organisms. WRN has been hypothesized to participate in homologous recombinational repair and in the processing of stalled replication forks.[40] Our previous study demonstrated that WRN-1 is able to unwind Holliday junctions, D-loops, and three-way junctions (resembling a synthetic replication fork). These structures are intermediates in homologous DNA recombination, replication fork regression, or stalled replication forks. In addition, we observed colocalization of WRN-1 and Rad51 foci (manuscript in preparation). Rad51 is a recombinase involved in nucleoprotein filament formation with ssDNA, and together with RPA, Rad51 promotes homologous pairing and strand exchange.[41,42] Thus, we propose that WRN-1 may function in homologous recombination or at stalled replication forks.

Another possible role of WRN-1 is in the DNA damage checkpoint. A recent study found that WRN-1 participates alongside RPA-1 in the initial stages of checkpoint activation induced by inhibition of DNA replication and ionizing radiation. Similarly, WRN is involved in the DNA damage checkpoint response to HU, DNA ICL, and CPT.[43] Furthermore, RPA has been implicated in signaling at stalled replication forks where the accumulated ssDNA is bound by RPA, creating a signal for activation of the ataxia-telangiectasia and rad3-related (ATR)-dependent checkpoint response.[44−46] We recently observed that WRN-1 functions downstream of RPA-1 in the checkpoint response to CPT (manuscript in preparation).

Further experiments using in vitro reconstitution of checkpoint signaling are needed to determine how DNA helicases participate in the DNA damage response and to assess the relevance of DNA helicases to aging of cells and organisms.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

A figure showing colocalization of WRN-1 and RPA-1 in response to CPT in mitotic germline cells from *C. elegans*. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Department of Life Sciences, University of Ulsan, Ulsan, Korea. Fax: +82-52-259-1694. Telephone: +82-52-259-2359. E-mail: bbccahn@mail.ulsan.ac.kr.

Present Address

⊥Department of Biochemistry and Molecular Biolgoy, Virgina Commonwealth University, Richmond, VA 23298-0614.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

RPA, replication protein A; WRN, Werner syndrome protein; WS, Werner syndrome; dsDNA, double-stranded DNA; nt, nucleotide; CPT, camptothecin; HU, hydroxyl urea; ICL, interstrand cross-link; DSB, double-strand break; ssDNA, single-stranded DNA; SD, standard deviation; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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