Identification of a Coiled Coil in Werner Syndrome Protein That Facilitates Multimerization and Promotes Exonuclease Processivity*

Received for publication, March 18, 2010, and in revised form, May 14, 2010 Published, JBC Papers in Press, June 1, 2010, DOI 10.1074/jbc.M110.124941

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Werner syndrome (WS) is a rare progeroid disorder characterized by genomic instability, increased cancer incidence, and early onset of a variety of aging pathologies. WS is unique among early aging syndromes in that affected individuals are developmentally normal, and phenotypic onset is in early adulthood. The protein defective in WS (WRN) is a member of the large RecQ family of helicases but is unique among this family in having an exonuclease. RecQ helicases form multimers, but the mechanism and consequence of multimerization remain incompletely defined. Here, we identify a novel heptad repeat coiled coil region between the WRN nuclease and helicase domains that facilitates multimerization of WRN. We mapped a novel and unique DNA-dependent protein kinase phosphorylation site proximal to the WRN multimerization region. However, phosphorylation at this site affected neither exonuclease activity nor multimeric state. We found that WRN nuclease is stimulated by DNA-dependent protein kinase independently of kinase activity or WRN nuclease multimeric status. In addition, WRN nuclease multimerization significantly increased nuclease processivity. We found that the novel WRN coiled coil domain is necessary for multimerization of the nuclease domain and sufficient to multimerize with full-length WRN in human cells. Importantly, correct homomultimerization is required for WRN function in vivo as overexpression of this multimerization domain caused increased sensitivity to camptothecin and 4-nitroquinoline 1-oxide similar to that in cells lacking functional WRN protein.

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§ The abbreviations used are: WS, Werner syndrome; DNA-PK, DNA-dependent protein kinase; WRN-exo, WRN exonuclease domain; DNA-PKcs, catalytic DNA-PK subunit; AMP-PNP, adenosine 5’-(β,γ-imino)triphosphate; WRN, Werner syndrome protein; BLM, Bloom syndrome protein.

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### Analysis

The primary focus of the research described in this document is the identification of a coiled coil region in the Werner syndrome protein (WRN) that facilitates multimerization and promotes exonuclease processivity. The study was supported by national Institutes of Health grants and other funding bodies.

**Key Points**

1. **Werner Syndrome (WS):**
   - A rare progeroid disorder characterized by genomic instability, increased cancer incidence, and early onset of aging pathologies.
   - Unique among early aging syndromes for having developmentally normal individuals.
   - Phenotypic onset is in early adulthood.
   - The protein defective in WS (WRN) is a member of the large RecQ family.

2. **Identification of Coiled Coil Region**
   - A novel heptad repeat coiled coil region was identified between the WRN nuclease and helicase domains.
   - This region facilitates multimerization of WRN.

3. **Multimerization and Processivity**
   - Multimerization significantly increased nuclease processivity.
   - Correct homomultimerization is essential for WRN function.

4. **Phosphorylation**
   - Phosphorylation at a novel site proximal to the WRN multimerization region did not affect exonuclease activity or multimeric state.
   - Stimulation of WRN nuclease by DNA-dependent protein kinase occurred independently of kinase activity.

5. **Functional Implications**
   - Overexpression of the novel WRN coiled coil domain caused increased sensitivity to camptothecin and 4-nitroquinoline 1-oxide.
   - The novel region divergently and may endow the diversity of functionalities to different RecQ family members.

**Conclusion**

The identification of a coiled coil region in WRN that facilitates multimerization and promotes exonuclease processivity is significant for understanding the mechanisms underlying WRN function and the diversity of RecQ family members.

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### References

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DNA-PK is then able to phosphorylate serine and threonine residues on a wide variety of substrates in vitro (19, 20). The four in vivo DNA-PK substrates that have been identified so far are DNA-PK itself, XRC4, histone H2AX, and WRN (7, 21, 22). Full-length WRN is also observed to assemble with DNA-PK on DNA, and WRN activity is regulated by the Ku70/80 subunit and the DNA-PK holoenzyme (7, 23–25), implicating WRN in DNA-PK-mediated DNA repair functions. Moreover, non-homologous end joining-mediated repair in WS cells exhibits extensive deletions, suggesting perhaps that another, less regulated exonuclease substitutes for WRN in these cells (26).

Here, we characterize the unique amino-terminal region of WRN and identify a specific multimerization region between the exonuclease and helicase domains near the amino terminus of WRN. Absence of this multimerization region altered the multimeric state of WRN-exo constructs and reduced nuclease processivity, resulting in pausing/terminating at specific sites on double-stranded DNA substrates. Expression of a small WRN fragment containing this region was sufficient to assemble heteromultimers with full-length WRN in human cells. Moreover, heteromultimer formation disrupted WRN function, causing sensitivity to camptothecin and 4-nitroquinoline 1-oxide similar to that observed in WS cells. The multimerization region also contains one unique DNA-PK phosphorylation site, but surprisingly, phosphorylation did not affect exonuclease processivity or multimerization state in vitro. However, interactions with DNA-PK increased nuclease processivity in WRN-exo constructs irrespective of the multimerization region, suggesting interactions with the core nuclease domain that stabilize WRN-exo interactions with DNA substrates. Sequence inspection of the multimerization region revealed a 50-amino acid heptad repeat coiled coil motif, also conserved in human RecQ1, with sequences most consistent with trimeric 50-amino acid heptad repeat coiled coil structures. Our results identify a novel domain functional in WRN multimeric assembly that gives insight into the organization and architecture of the WRN protein.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—PCR primers were designed based on limited proteolysis results and secondary structure predictions. PCR was performed with Pfu polymerase, the resulting PCR products were cloned into pET23 expression vector (Novagen), and the sequence was confirmed. Clones were grown in liquid culture and induced with 100 mM isopropyl β-D-galactopyranoside in midlog phase, harvested after 4–12 h, and lysed by sonication, and the extract was cleared by centrifugation. Expression and solubility were evaluated by Coomassie Blue-stained SDS-PAGE. Clones showing high levels of soluble protein expression were scaled up to multiple liter scale, and the proteins were purified to near homogeneity with nickel-nitrilotriacetic acid (GE Healthcare), Mono Q (GE Healthcare), and Superdex S-200 gel filtration (GE Healthcare) chromatography.

**Antibodies**—Antibodies recognizing WRN amino termini were raised in New Zealand white rabbits against purified recombinant WRN(1–333) using standard protocols (Apex Materials Safety). Antiserum from two rabbits was tested for reactivity on WRN(38–236), WRN(1–236), and WRN(1–333); antiserum 10061 recognized epitopes within WRN(236–333). Polyclonal rabbit antibodies recognizing polyhistidine (H-15) were purchased from Santa Cruz Biotechnologies.

**Kinase Assays**—Kinase assays were carried out as described previously (7) using ~0.5 pmol of DNA-PK, 1 pmol of WRN, and 0.75 μg of sheared salmon sperm DNA. Reactions were separated by 7.5% SDS-PAGE, visualized, and quantified using a PhosphorImager and ImageQuant software from GE Healthcare.

**Gel Filtration and Dynamic Light Scattering**—The purified WRN(38–236), WRN(1–236), and WRN(1–333) proteins were loaded onto a Superdex S-200 (GE Healthcare) gel filtration column equilibrated with 150 mM sodium/potassium phosphate, pH 7.5, 500 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol buffer at a flow rate of 0.3 ml/min at 4 °C. The protein mass standards were purchased from GE Healthcare and were run under identical chromatographic conditions for column calibration (ferritin, 440 kDa; aldolase, 158 kDa; albumin, 67 kDa; chymotrypsinogen, 25 kDa). Dynamic light scattering measurements were carried out using a DynaPro 99 dynamic light scattering instrument from Protein Solutions. 250-μl samples of WRN(38–236), WRN(1–236), and WRN(1–333) in gel filtration buffer were filtered through a Whatman membrane of 0.2-μm pore size, manually injected into the 16 °C flow cell, and illuminated with an incident beam from a 25-milliwatt, 790 nm solid-state laser.

**Exonuclease Assays**—Exonuclease assays were carried out under conditions reported previously (7). Briefly, a 35-bp double-stranded DNA oligonucleotide with five nucleotide single strand extensions on both 5’ termini was labeled on one strand and annealed (5’-GCC GCA AAT CAA CAC GTT GAC TAC CGT CTT GAG GCA GAG T; 5’-CGG GGA CTC TGC CTC CAG AGC GTA GTC AAC GTG TTG ATT T). Approximately 300 fmol of probe was incubated for 30 min at 37 °C in 50 mM HEPES, pH 7.5, 50 mM KCl, 10 mM MgCl2, 1 mM dithiothreitol with the indicated amount of protein(s). Reaction products were resolved on 16% or 20% polyacrylamide, Tris borate-EDTA gels containing 8.3 M urea and visualized by phosphorimaging and/or autoradiography.

**Immunoprecipitation**—HeLa cells stably expressing FLAG-WRN(250–366) were lysed in buffer containing 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.05% SDS, 0.1% sodium deoxycholate, and protease inhibitor mixture (Complete, Roche Applied Science) on ice for 20 min. Lysate was cleared by centrifugation at 20,000 × g for 10 min and then precleared with mouse IgG for 1 h at 4 °C. The pre-cleared lysate was then incubated with anti-FLAG antibody (Sigma-Aldrich) and WRN monoclonal antibody (raised against 940–1432 amino acid region of WRN) for 4 h at 4 °C, and antibody-antigen complex was recovered by using protein A/G-agarose beads (Roche Applied Science) for 4 h at 4 °C. Precipitates were washed three times with immunoprecipitation buffer, then eluted in SDS-PAGE loading buffer, and analyzed by Western blotting.

**Clonogenic Survival Assays**—Parental HeLa and HeLa cells stably expressing FLAG-WRN(250–366) were plated in triplicate (3 × 10²–2 × 10³ cells/25-cm² flask) and incubated for 12–18 h. Cells were then treated with varying doses of 4-nitroquinoline 1-oxide, hydroxyurea, and camptothecin. Cells were
washed twice, trypsinized, counted, and replated in three 10-cm² dishes at 1, 24, and 72 h after 4-nitroquinoline 1-oxide, hydroxyurea, and camptothecin treatments, respectively. After a 12-day incubation, dishes were stained with crystal violet (0.5% crystal violet, 1% formaldehyde, 1× phosphate buffered saline), and survival was scored by quantifying colonies. Survival curves were generated from three independent experiments with colony numbers normalized to sham-treated controls.

WRN Sequence Analysis—A search for homologues of human WRN (UniProt accession number Q14191) was conducted using PSI-BLAST (27). Alignments were performed using the BioEdit (Ibis Therapeutics, Carlsbad, CA) and ClustalW alignment software followed by manual adjustment. Analysis of coiled coil motifs was conducted using Coils (28) and Marcoil (29).

RESULTS

Identification of WRN Exonuclease Domain Boundaries and Recombinant Protein Production—Our previous studies defining the crystal structure of the core WRN exonuclease, amino acids 38–236 (WRN(38–236)), determined that this domain was monomeric (14). However, a slightly larger construct, WRN(1–333), was reported to form a high order complex (30). To identify potential multimerization regions and define domain boundaries, we purified and characterized WRN(1–333). Purified recombinant protein from E. coli cells was subjected to limited proteolysis with either trypsin or chymotrypsin and visualized by Coomassie Blue-stained SDS-PAGE (Fig. 1A). Mass spectrometry and amino terminus sequencing were used to delineate the proteolytic fragments and identify domain boundaries. Our data indicated two major proteolytic cleavage sites located approximately at amino acids 38 and 236 of the WRN(1–333) construct. These results guided the construction of WRN(1–236), was reported to form a high order complex (30), whereas amino acids 236–333 constitute a carboxyl-terminal extension from the core exonuclease that largely mediates WRN nuclease multimerization. The first 38 amino acids of WRN play a relatively minor role in multimerization, whereas amino acids 236–333 constitute a carboxyl-terminal extension from the core exonuclease that largely mediates WRN nuclease multimerization.

WRN Contains a Coiled Coil Motif That Self-assembles—Inspection of the human WRN amino acid sequence revealed an amino acid pattern indicative of a heptad coiled coil motif within the identified multimerization region. Coiled coil motifs often function to mediate protein-protein interactions and protein multimerization (31). Alignment of the human WRN sequence with homologous proteins having both helicase and exonuclease domains revealed conservation of the coiled coil sequence with homologous proteins having both helicase and exonuclease-D domains schematically illustrated.
**WRN Multimerization Domain**

![Graph](image)

**TABLE 1**

Dynamic light scattering measurements for three WRN nuclease constructs

| WRN   | Molecular weight | Population | Radius | Calculated mass |
|-------|------------------|------------|--------|-----------------|
| aa    | % (nm) | kDa | 1–333 | 39,200 | 97.4 | 4.53 | 113 |
| 1–236 | 27,800 | 86.1 | 2.49 | 26.4 |
| 38–236 | 23,600 | 91.6 | 2.49 | 26.4 |

To further evaluate the functionality of the multimerization region, we constructed a bacterial expression vector fusing a polyhistidine tag to WRN amino acids 228–333. This construct included the entirety of the predicted coiled coil region. Initial expression screening surprisingly revealed a ladder of bands visible on SDS-PAGE with an apparent periodicity of increasing mass in addition to the expected 17-kDa band (Fig. 3B, left). To test whether these bands were multimers of the WRN coiled coil that were resistant to SDS denaturation, we carried out Western blots using antibodies recognizing a WRN epitope between amino acids 228 and 333 (Fig. 3C). These data show that WRN(228–333) forms multimers of the 17-kDa construct that are resistant to disassembly on SDS-PAGE (Fig. 3C). To confirm this unexpected result, we repeated this experiment with commercial antibodies recognizing polyhistidine (Fig. 3D). These data confirm that WRN(228–333) forms multimers that are resistant to SDS denaturation. Variations in buffers and expression conditions were found to perturb the distribution of multimeric forms, but no conditions tested dissociated all multimers (data not shown). Although the monomeric (17-kDa) form is the predominant species, other discrete bands are clearly visible on the Western blots. Notably, higher mass forms migrating at approximate masses of 51 and 102 kDa, which correspond to trimeric and hexameric forms, respectively, and no larger multimers were observed (Fig. 3, C and D).

**WRN Coiled Coil Domain Assembles with Full-length WRN in Human Cells**—To test the relevance of the identified coiled coil domain to WRN assembly in human cells, we constructed a mammalian expression vector containing the identified coiled coil motif (amino acids 250–366) fused to a FLAG epitope tag. This expression vector was stably transfected into HeLa cells, and WRN was immunoprecipitated from cleared cellular extracts. Immunoprecipitation with anti-FLAG antibodies recognizing FLAG-WRN(250–366) precipitated the associated full-length WRN, indicating that a significant proportion of these proteins assemble into heteromultimers in vivo (Fig. 4A, lane 3). Likewise, immunoprecipitation of extracts with antibodies recognizing WRN epitopes not present on the recombinant FLAG-WRN(250–366) effectively co-precipitated the WRN(250–366) well above background levels (Fig. 4A, lane 4). Taken together, these data indicate that the coiled coil region of WRN is sufficient to facilitate multimerization with full-length WRN in human cells.

**WRN Multimerization Domain Disrupts WRN Function in Vivo**—Cells lacking functional WRN protein have a markedly increased sensitivity to the DNA-damaging drugs camptothecin and 4-nitroquinoline 1-oxide (33–37). To determine the influence of WRN(250–366) expression on WRN function, we carried out toxicity assays using cells expressing FLAG-WRN(250–366). We found that recombinant expression of the WRN multimerization domain causes a distinctly elevated sensitivity to both camptothecin and 4-nitroquinoline 1-oxide (Fig. 4, A and C). Notably, expression of the multimerization domain did not increase sensitivity to hydroxyurea, suggesting that this effect is not a generalized loss of cellular fitness (Fig. 4D). These data are most consistent with heteromultimeric forms of WRN and WRN(250–366) having compromised function in vivo.

**DNA-PKcs Phosphorylates a Single Serine on Amino Terminus of WRN**—The DNA-PK is known to influence the helicase and nuclease activities of WRN through interaction and phosphorylation of the full-length WRN (7, 23). We therefore tested whether DNA-PK phosphorylation events occur on the nuclease domain. *In vitro* kinase reactions using radioactive
[32P]ATP were carried out with purified WRN-exo proteins and resolved by SDS-PAGE (Fig. 5A). We observed robust radioactive labeling of both full-length WRN and WRN(1–333) in sharp contrast to the marginal radioactive labeling of WRN(1–236) in equivalent DNA-PK kinase reactions (Fig. 4A). Inspection of the 97 amino acids absent from WRN(1–236) but included in WRN(1–333) revealed 11 serine and five threonine residues, none of which were within a canonical (S/T)Q recognition motif for DNA-PK (38). Mass spectrometry of in vitro phosphorylated WRN(1–333) revealed a nearly saturated single phosphorylation on a peptide that contained two serine residues, Ser-319 and Ser-323. Site-directed mutagenesis was conducted on either of these two sites to establish the specific phosphorylation sites of WRN(1–333). The mutant proteins were incubated with either DNA-PK or a mixture of commercially available phosphatases (λ and PP1). The reaction products were analyzed by size exclusion chromatography, and the elution profiles were inspected for changes in WRN(1–333) multimeric state. Kinase reactions were validated by inclusion of radioactive ATP and incorporation of [32P]P (data not shown). We observed that irrespective of phosphorylation status WRN(1–333) eluted as a single symmetrical peak at a volume consistent with a trimeric form (Fig. 6A). Therefore, DNA-PK phosphorylation at serine 319 does not alter the multimeric state of this construct in vitro.

Interactions with DNA-PK, but Not Phosphorylation, Alter WRN Exonuclease Activity—We next evaluated the influence of phosphorylation on WRN exonuclease activity. WRN(1–333) was assayed for exonuclease activity in the presence of DNA-PK and either ATP, the non-hydrolyzable ATP analogue AMP-PNP, or phosphatase (Fig. 6B, lanes 1–8). As previously reported (7), the addition of Ku70/80 stimulated exonuclease activity, and the addition of DNA-PKcs by itself reduced nuclease processivity (Fig. 6B, lanes 1–8). Importantly, in the presence of holo-DNA-PK (Ku70/80 + DNA-PKcs), digestion patterns were essentially unchanged whether buffer alone, ATP, AMP-PNP, or λ-phosphatase was added (Fig. 6B, lanes 4–7). These data unambiguously show that neither the phosphorylation status of WRN(1–333) nor the kinase activity of DNA-PK alters exonuclease activity under these experimental conditions. As expected, ATP, AMP-PNP, and phosphatase additions did not dramatically alter the DNA digestion patterns of WRN(1–236) and WRN(38–236) constructs as these constructs lack DNA-PK phosphorylation sites (Fig. 6B, lanes 12–15 and 20–23). Consistent with our previous work with full-length WRN, all WRN-exo constructs were stimulated by Ku70/80 and holo-DNA-PK and diminished by DNA-PKcs in a kinase-independent manner (7). Taken together, these data indicate that protein-protein interactions between WRN-exo and DNA-PK alter exonuclease processivity independently of DNA-PK kinase activity, WRN-exo multimeric status, or phosphorylation of the WRN nuclease.

Multimeric State of WRN Constructs Influences Nuclease Processivity—In the experiments above, we noted a marked difference in the exonuclease processivity between the three different perturbed the multimeric state of WRN(1–333). Equivalent quantities of WRN(1–333) were incubated with either DNA-PK or a mixture of commercially available phosphatases (λ and PP1). The reaction products were analyzed by size exclusion chromatography, and the elution profiles were inspected for changes in WRN(1–333) multimeric state. Kinase reactions were validated by inclusion of radioactive ATP and incorporation of [32P]P (data not shown). We observed that irrespective of phosphorylation status WRN(1–333) eluted as a single symmetrical peak at a volume consistent with a trimeric form (Fig. 6A). Therefore, DNA-PK phosphorylation at serine 319 does not alter the multimeric state of this construct in vitro.

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FIGURE 4. WRN coiled coil domain multimerizes with WRN in vivo and alters WRN function. A, immunoprecipitations (IP) of extracts from HeLa cells overexpressing FLAG-WRN(250–366); antibodies used for immunoprecipitation (above) and proteins detected are indicated (left), full length WRN (WRN-FL). B–D, colony formation assays comparing the survival of parental HeLa cells (solid black circles) with the survival of HeLa cells expressing FLAG-WRN(250–366) (open circles) to the indicated doses of camptothecin (B), 4-nitroquinoline 1-oxide (C), and hydroxyurea (D). All assays were carried out in triplicate; the average values are plotted, and the standard deviation (error bars) is indicated.

FIGURE 5. DNA-PK phosphorylates WRN-exo specifically at Ser-319 within the multimerization region. A, in vitro kinase assays containing ~500 ng of DNA-PK and [32P]ATP resolved by SDS-PAGE. Lane 1, DNA-PK reaction alone; lane 2, DNA-PK reaction with ~50 ng of full-length WRN; lane 3, DNA-PK reaction with ~1 µg of WRN(1–333); lane 4, DNA-PK reaction with ~1.5 µg of WRN(38–236). The Coomassie Blue-stained gel (left panel) and the phosphorimage of the same gel (right panel) are shown. Reaction component proteins are indicated (left). B, DNA-PK phosphorylates the WRN nuclease domain specifically at serine 319. Partially purified WRN(1–333), either wild type (WT) Ser-319, S319A mutation, or S323A mutation, was incubated and resolved on SDS-PAGE as above, stained with Coomassie Blue, and exposed to a phosphorimage screen (top panel) or photographed (middle panel), or a parallel gel was transferred to nitrocellulose and probed with an antibody recognizing the nuclease domain of WRN (bottom panel). The proximal amino acid sequence is shown with Ser-319 (large red) and Ser-323 (small red) indicated.

WRN-exo constructs (Fig. 7A). At the lowest concentrations of enzyme, a clear difference in digestion pattern was evident with the same pause sites being apparent in all cases (Fig. 7A, lanes 1, 4, and 7). Although all three forms of the nuclease can digest nearly all of the initial substrate, we observed obvious differences in the accumulation of intermediate products (Fig. 7, P1 and P2). Importantly, this change in processivity trends with the established multimeric character of the three WRN-exo constructs (Figs. 2 and 7A). Most notably, the WRN(38–236) construct appears to initiate as efficiently as the other constructs as evidenced by the loss of the full-length substrate; however, nearly all of the products accumulated at the noted pause sites even at the highest enzyme concentration (Fig. 7A, lanes 7–9). In contrast, stoichiometrically equivalent amounts of WRN(1–333) and WRN(1–236) readily traversed the first pause site, and in the case of WRN(1–333), the second site was also readily traversed. Thus, these data indicate that the multimerization region via higher order assembly provides increased nuclease processivity and progression through potential pause sites.

DISCUSSION

Members of the RecQ family are reported to exist in different multimeric forms, and the relationships between multimerization states and functionality remain an active area of research. Much of the biochemical and biophysical data support the idea that multimeric forms of RecQ helicases are biologically relevant. The E. coli RecQ was observed to hydrolyze ATP with a Hill coefficient of 3, implying that multiple ATPase sites act during DNA strand separation (39). The Drosophila melanogaster RecQL5 forms homo-oligomers (40), electron microscopy shows BLM in hexameric or tetrameric forms (41), amino-terminal fragments of BLM form hexamers and dodecamers (42), and oligomeric forms of WRN bind replication forks and Holliday junctions (43). Evidence from size exclusion chromatography and atomic force microscopy also indicates that full-length WRN assembles as trimers and hexamers in the presence of DNA (30, 44). Human RecQ1 helicase forms oligomeric structures that are necessary for its Holliday junction resolution activity (45). However, there are reports dissenting from the mutimeric view of RecQ helicases, including biochemical characterization of E. coli RecQ concluding that this microbial enzyme functions as a monomer (46); also the recombinant BLM helicase domain is monomeric and catalytically active (47). Unfortunately, direct structural insight into the spatial arrangement of RecQ domains in multimeric forms is limited as all available high resolution structures for RecQ proteins have been of truncated monomeric forms. These include crystal structures of truncated cores from E. coli RecQ (48) and human RecQL1 (49) helicases, the WRN-exo domain (14), and the carboxyl-terminal winged helix and HRDC domains from various RecQ proteins (50–54). However, the WRN-exo crystal structure shares significant homology with a hexameric ring-structured nuclease from Arabidopsis thaliana (Protein Data Bank code 1VK0). We found that superimposition of WRN-exo onto the A. thaliana ring produces a hexameric model with active sites oriented toward the center of a ring with an opening large enough to accommodate double-stranded DNA (14). Regardless, there remains substantive variability in the data regarding
the multimeric status of various RecQ protein family members, and no unifying hypothesis has thus far been put forth.

Our studies on the WRN amino-terminal region revealed that the ~100 amino acids immediately downstream of the core exonuclease are required for oligomerization. Moreover, the multimeric state of the nuclease influenced processivity with higher order assemblies showing diminished “pausing” relative to the monomeric form (Fig. 7A). Surprisingly, recombinant expression of the multimerization region resulted in oligomeric forms that were resistant to the typically denaturing conditions of SDS-PAGE (Fig. 3). In addition, overexpression of the WRN multimerization region in human cells revealed that this region is sufficient to facilitate oligomerization with full-length endogenous WRN in vivo (Fig. 4A). Importantly, this hetero-multimerization caused an apparent disruption of WRN function within the cell (Fig. 4). Taken together, these data suggest that the multimerization domain identified here has a critical function in oligomerization that influences WRN function in vivo.

Inspection of the amino acid sequence in the identified multimerization region revealed the presence of a heptad coiled coil motif spanning approximately 50 amino acids (Fig. 3A). Following a relatively simple set of rules, the specific amino acids occupying the 1 and 4 positions of a heptad repeat dictate whether a dimer, trimer, or tetramer is formed (31, 32). Consistent with our biochemical data, the WRN heptad sequence shows a strong bias for trimer formation with six of the eight position 1 amino acids being occupied by leucine. Such a three-stranded coiled coil may account for the robust affinity observed with WRN multimerization constructs under denaturing conditions (Fig. 3).

Notably, we identified a similar heptad repeat sequence in the amino terminus of human RecQ1 (Fig. 3A). Interestingly, truncated RecQ1 helicase lacking this coiled coil region cannot form oligomers nor can it catalyze Holliday junction resolution (45). This suggests a common mechanism for multimerization among RecQ helicases that is critical for function. Unlike the RecQ1 heptad repeat, WRN repeats have a conserved break in the heptad pattern at amino acids 278 and 281 (Fig. 3A).

Such breaks in coiled coil motifs are often referred to as a stutter or skip and are thought to be associated with a hinge or flexible region along the coiled coil (55). Variability among these simple heptad sequence motifs between different RecQ family members may reflect the variability in oligomeric forms and function between RecQ members within and between species. Moreover, the flexibility of coiled coils to facilitate various multimeric states may permit switching between multimeric states and/or switching of subunits, thereby contributing to the functional plasticity of enzymes containing these domains.

DNA-PK is an abundant human nuclear kinase that functions in DNA double strand break repair, has inherent lyase activity, and has telomere capping functions (15–17, 56, 57). It functionally and physiologically interacts with WRN via regulation and phosphorylation of WRN, and WRN-deficient cells show...
aberrant non-homologous end joining repair junctions (7, 23–26). Here, we identify a singular and unique DNA-PK phosphorylation site on the WRN(1–333) nuclease at serine 319. We found that phosphorylation at this site does not alter the multimerization or activity of the trimeric WRN(1–333) under the conditions tested. Such phosphorylations can control partner protein interactions, assembly, and allosteric communication as with the Nbs1 component of the Mre11-Rad50-Nbs1 complex (58, 59). Our data suggest that phosphorylation at WRN Ser-319 may act in altering remote activities of the full-length protein or influencing communication between domains or may play non-enzymatic roles such as facilitating subcellular localization of WRN.

Regardless, our data reveal that DNA-PK alters WRN exonuclease activity in a manner independent of the multimerization domain or DNA-PK kinase activity likely through direct protein–protein interactions. Moreover, these putative interactions must be at least partly contained within WRN(38–236) as both Ku and DNA-PKcs influenced nuclease activity of this construct. Interestingly, both DNA-PK interactions and multimerization increased WRN-exo processivity, suggesting the possibility that increased residence on double-stranded DNA substrates, not phosphorylation, may be at least in part the mechanism that modulates exonucleolytic processivity. Such direct interface exchanges or handoffs that avoid the release of toxic and mutagenic intermediates are proposed to occur for many DNA repair nucleases such as APE1 (60), FEN-1 (61), endonuclease IV (62), Mre11 (63), and endonuclease V (64). Furthermore, such handoff interactions may channel DNA damage from one repair pathway to another as shown by structural and genetic results on the alkylation damage defense by the protein Atl, which provides damage protection without doing any repair (65). The role of such a DNA handoff process between WRN multimers and/or partner proteins is an area of ongoing investigation.

Overall, we have herein built upon previous work to identify a novel coiled coil domain that facilitates multimerization and bridges the helicase and nuclease domains of WRN. This domain not only plays a critical role in WRN architecture but also contains a single novel DNA-PK phosphorylation site (Fig. 7B). Although no enzymatic function is directly ascribed to the WRN coiled coil domain, we found that it indirectly influences WRN nuclease activity and WRN function in vivo and has broad architectural implications.

Our findings suggest that WRN may exist as a dimer of trimers having two stacked rings, one harboring the nuclease activity and the other harboring the helicase activity (Fig. 7C). A dimer of trimers organization may allow “rings” of this enzyme to open and close to encircle DNA substrates and regulate enzymatic residence on substrates. Notably, such DNA-mediated assembly is reported for WRN nuclease hexamers (44). An extended coiled coil between these functional regions may permit translational freedom for the respective enzymatic activities and at the same time limit the distance between them (Fig. 7C). Such flexibility may also allow cooperation in DNA end processing between WRN and the Ku dimer acting as an apurinic/apyrimidinic lyase (56). Although this model is hypothetical, it provides testable ideas as the identification of the coiled coil domain between the known enzymatic domains of WRN points to a limited number of architectural arrangements for this enzyme. Such structural insights into Xeroderma pigmentosum group D (XPD) helicase mutations associated with aging versus cancer disorders provided testable predictions regarding mutants impacting excess cell death and aging in the organism versus those causing genetic instability and cancer (66). For the Xeroderma pigmentosum group B helicase, such implications from the structures and biochemistry provided key insights into functional motifs and recruitment that were then tested and validated in human cells (67, 68). Here, insights into the WRN helicase/exonuclease from its structure and biochemistry may therefore lead to a better understanding of its interactions and function, which so profoundly influence the human aging process.

Acknowledgments—We thank David King, Howard Hughes Medical Institute at the University of California Berkeley, for assistance with the mass spectrometry, Robert P. Rambo and Kevin N. Dyer for aiding with experiments and helpful discussions, and Misako Kawahara Stillion for comments on the manuscript.

REFERENCES
1. Muftuoglu, M., Oshima, J., von Kobbe, C., Cheng, W. H., Leistritz, D. F., and Bohr, V. A. (2008) Hum. Genet. 124, 369–377
2. Ozgenc, A., and Loeb, L. A. (2006) Genome Dyn. 1, 206–217
3. Martin, G. M., Sprague, C. A., and Epstein, C. J. (1970) Lab. Invest. 23, 86–92
4. Salk, D., Au, K., Hoehn, H., and Martin, G. M. (1981) Cytogenet. Cell Genet.
