The Werner Syndrome Gene Product Co-purifies with the DNA Replication Complex and Interacts with PCNA and Topoisomerase I*

Michel Lebel‡§, Elisa A. Spillare¶, Curtis C. Harris¶, and Philip Leder‡**

From the ‡Department of Genetics, Harvard Medical School, the §Howard Hughes Medical Institute, Boston, Massachusetts 02115, and the ¶Laboratory of Human Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892-4255

Werner syndrome (WS) is a recessive disorder characterized by genomic instability and by the premature onset of a number of age-related diseases. To understand the molecular basis of this disease, we deleted a segment of the murine Wrn gene and created Wrn-deficient embryonic stem (ES) cells. At the molecular level, wild type—but not mutant—WS protein co-purifies through a series of centrifugation, chromatography, and sucrose gradient steps with the well characterized 17 S multiprotein DNA replication complex. Furthermore, wild type WS protein co-immunoprecipitates with a prominent component of the multiprotein replication complex, proliferating cell nuclear antigen (PCNA). In vitro studies also indicate that PCNA binds to a region in the N terminus portion of the WS protein containing a potential 3′-5′ exonuclease domain. Finally, human WS protein also co-immunoprecipitates with both PCNA and topoisomerase I. These results suggest that the WS protein interacts with several components of the DNA replication fork.

Werner syndrome (WS) is a rare disorder characterized by the premature onset of a number of processes associated with aging (1, 2). In addition, the proliferative life span of WS fibroblasts is reduced compared with age-matched controls (3–5). WS cells from patients also exhibit genomic abnormalities such as variegated chromosomal translocations and deletions (6, 7). Most recently, WS cells have been shown to have an attenuation of a p53-dependent apoptotic pathway (8).

The WS gene (WRN) product contains seven helicase consensus domains that are 34–38% identical to the Escherichia coli RecQ gene (9, 10) and to the putative yeast helicase Sgs1p (11, 12). The Sgs1p is known to interact with types I and II topoisomerase, and several of its specific components.

**Experimental Procedures**

Protein Analysis—Generation and maintenance of the wild type and homozygous mutant embryonic stem cells have been described previously (17). Protein extraction, immunoprecipitations, and Western blotting analyses were performed as described (8, 18). A rabbit polyclonal antibody was raised against the first 42 amino acid residues of the N terminus portion of WS protein (Covance Research Products Inc.) as described (17). The monoclonal antibody against PCNA (AB-1) was purchased from Oncogene Research and used as indicated by the manufacturer. The polyclonal antibody against topoisomerase I was purchased from Immunovision. The antibody against the human WRN and the WRN peptide was purchased from Santa Cruz Biotechnology. All horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham Pharmacia Biotech. Proteins on Western blots were visualized with an ECL kit (Amersham Pharmacia Biotech).

Affinity Purification of Recombinant Protein or “Pull Down” Assay—Several GST fusion proteins were constructed for the pull down or affinity purification assay. Table I gives a list of the constructs. Plasmids were transfected into BL21 bacteria for fusion protein production.

Approximately 108 ES cells of the indicated genotype were lysed in 10 mM Tris-HCl, pH 7.5, 1% (v/v) Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor mixture (Roche Molecular Biochemicals) at 4 °C for 20 min. Cells were sonicated 5 s, and cell debris were spun down. Affinity matrices were prepared by immobilizing GST alone or GST-fusion proteins on glutathione-Sepharose beads (Amersham Pharmacia Biotech) as described (19). Freshly prepared cell lysates were incubated with the affinity matrices for 2 h at 4 °C. After extensive washing with the lysis buffer, bound proteins were released by boiling in SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. Proteins were also visualized by Coomasie staining when indicated.

The binding of WS protein to PCNA was detected by cleaving the WS portion of the GST-WS fusion protein (residues 45 to 657 of WS peptide) with thrombin in phosphate-buffered saline. The beads were centrifuged, and the supernatant containing the WS peptide was incubated with heart muscle kinase and [γ-32P]ATP as described by the manufacturer (Sigma Co.). Approximately 107 cpm of labeled WS peptide were incubated 2 h with different GST-PCNA affinity beads. The beads were washed five times with phosphate-buffered saline, and 2× gel loading buffer was added to the beads for gel protein analysis.

Fractionation Procedure—ES cells were fractionated as described (20). Five grams of frozen pellet were thawed and resuspended in 3 volumes of buffer containing 50 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 5 mM MgCl2, 0.1 mM PMSF, and 1 mM dithiothreitol (DTT). The resus-

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** To whom correspondence should be addressed: Dept. of Genetics, Harvard Medical School, Howard Hughes Medical Institute, 200 Longwood Ave., Boston, MA 02115. Tel.: 617-432-7667; Fax: 617-432-7683.

† The abbreviations used are: WS, Werner syndrome; ES, embryonic stem; GST, glutathione S-transferase; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol.

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and made 2M in KCl. Polyethylene glycol was added to a final concentration for 30 min at 16,000 g. The clarified fraction was then dialyzed as described (20). The clarified fraction was collected separately. Mitochondria were removed from the cytosolic fraction by centrifugation at 27,000 g for 15 min. The resultant supernatant was then subjected to centrifugation at 100,000 × g for 60 min to remove microsomes. The post-microsomal supernatant was collected.

The crude nuclear extract was resuspended in 2 volumes of a buffer containing 50 mM Tris-HCl, pH 7.5, 0.15 M KCl, and 1 mM DTT. The resuspended nuclei were then centrifuged for 10 min at 500 g. The post-microsomal supernatant was collected.

Sucrose Sedimentation Gradient—A 9-ml 10–30% sucrose gradient in 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA-Na3, and 1 mM DTT was formed over a 1-ml cushion of 2 M sucrose in polyallomer tubes. A 0.5-ml aliquot of protein fraction was loaded onto the pre-formed gradient and centrifuged at 100,000 × g for 18 h at 4 °C. The material above the sucrose interphase was collected and designated the HS-4 fraction. The sucrose interphase fraction was collected and designated HSP-4.

RESULTS

A Deletion of a Portion of the WS Helicase Domain Affects Its Interaction with the DNA Replication Complex—The human WRN gene encodes a central motif composed of seven domains constituting the helicase signature. Several Werner syndrome patients have been reported to carry WS mutations involving these exons encoding helicase domains III and IV (21). The mouse WS protein also contains these seven helicase domains. To recapitulate part of the activity of this complex. Accordingly, analyses were performed to determine whether the mutant and the wild type WS proteins are found in sub-cellular fractions containing the well characterized multiprotein DNA replication complex. To demonstrate a potential association, fractionation of the WS ES cell proteins was performed according to a protocol that results in substantial purification of the multiprotein DNA replication complex (20). Briefly, extracted proteins were centrifuged onto a 2 m sucrose cushion and divided into an interphase fraction consisting of high molecular weight protein complexes (including the multiprotein DNA replication complex referred to as fraction HSP-4) and a supernatant fraction consisting of soluble proteins (fraction HS-4). Fig. 1A shows that the wild type WS protein is found in the HSP-4 complex fraction (+/+) lane, suggesting that the WS protein might be a part of a multiprotein complex. Most importantly, however, we see that the mutant WS protein is not detected in the complex fraction (Fig. 1A, −/− lane) as shown earlier (17). There is no significant decrease in helicase activity in the complex fraction from mutant cells compared with the fraction from the wild type cells (data not shown). This result may be because of other contaminating helicases during preparation of the fractions that are associated with DNA replication (22).

The complex fraction was further analyzed on a 10–30% sucrose gradient wherein the bulk of DNA polymerase activity migrates with the 17 S sucrose fraction containing the multiprotein DNA replication complex (data not shown) (20). The yeast 7 S alcohol dehydrogenase and the 17 S horse spleen apo-ferritin were used as protein markers on parallel gradients to assess the distribution of protein size for each gradient experiment. Western blot analyses were carried out to detect the WS protein in each fraction number of the sucrose gradient. In each experiment (repeated three times), most WS proteins were detected in fraction numbers 5 and 6 of the gradient (Fig. 1B) where the 17 S protein marker was also detected (asterisks in lanes 5 and 6) by Coomassie staining (data not shown). These data demonstrate that the wild type WS protein co-purifies with the 17 S multiprotein DNA replication complex and, therefore, might be a part of that complex. Moreover, the possibility that this association is a physiologic one is sug-
The WS Protein Interacts with PCNA—To identify components of the DNA replication complex which might bind to the WS protein, immunoprecipitation experiments were carried out on the complex fraction (HSP-4) with the antibody raised against the mouse WS protein. As expected, immunoprecipitation on the complex fractions followed by Western blot analysis with the same antibody showed a 170-kDa band for wild type (+/+), homozygous mutant (−/−), or no (no) total ES cell lysate. In the bottom panel, the proteins from a duplicated gel (as in the top panel) were transferred onto a membrane for immunoblot analysis with an anti-PCNA antibody. The arrows on the right indicate the positions of the fusion, the GST, and PCNA on the gel or the protein blot. A, association of PCNA with the N-terminal portion of the WS protein. The gray box represents the acidic domain, and the black boxes represent the seven helicase motifs of the WS protein. The numbers on the left are the amino acid residues of the GST-fusion peptides immobilized on the affinity matrix. The beads were incubated with total cell lysates of homozygous mutant (−/−), or no (no) total ES cell lysate. B, Coomassie staining of the GST-fusion proteins used for the pull down assay. In the top panel, GST and the GST-WS (residue 45 to 657 of WS protein) fusion peptide affinity matrices incubated with either wild type (+/+), homozygous mutant (−/−), or no (no) total ES cell lysate. In the bottom panel, the proteins from a duplicated gel (as in the top panel) were transferred onto a membrane for immunoblot analysis with an anti-PCNA antibody. The arrows on the right indicate the positions of the fusion, the GST, and PCNA on the gel or the protein blot. C, association of PCNA with the N-terminal portion of the WS protein. The gray box represents the acidic domain, and the black boxes represent the seven helicase motifs of the WS protein. The numbers on the left are the amino acid residues of the GST-fusion peptides immobilized on the affinity matrix. The beads were incubated with total cell lysates of homozygous mutant (−/−), or no (no) total ES cell lysate. The summary of the data shown in Fig. 2B, lane 2, further analyses were performed with fusion proteins containing different segments of the WS protein. Table I gives a list of all the fusion constructs used in this study. The summary of the data shown in Fig. 2C indicates that PCNA binds to a region of the WS protein containing amino acids 168 to 246.

The WS protein/PCNA interaction could be either a direct interaction or one mediated by associated protein(s). To begin to distinguish between these possibilities, an array of GST-PCNA fusion proteins was immobilized on beads and incubated 2 h with radioactively phosphorylated WS protein (amino acids 45 to 657) for pull down experiments. Fusion proteins from each construct were analyzed on a gel by Coomassie staining for quantification (Fig. 3A). The results in Fig. 3B indicate that the WS peptide binds directly to the PCNA protein. Moreover, it binds strongly to the C-terminal half of the PCNA protein (residue 160 to 261) but very weakly to the first 134 residues of PCNA (see Fig. 3C for summary of results).

The WS Protein Interacts with PCNA and Topoisomerase I—The blots from the pull down experiments were also probed with antibodies against the human topoisomerase I, 70-kDa RPA protein, and poly(ADP-ribose) polymerase (PARP). Antibodies against the human proteins were used because no antibody against the murine peptides was available. These proteins are known to be localized at the replication fork (23–26). Topoisomerase I and RPA were detected with the full-length GST-fusion proteins encoding segments of the WS protein were synthesized, associated with affinity matrices (beads), and used to pull down interacting proteins from whole cell lysates of wild type and homozygous mutant WS ES cells. The associated lysate proteins were then electrophoresed and identified on protein blots using antibodies against known proteins found at the replication complex. As shown in Fig. 2B, lane 2, a fusion construct containing amino acids 45 to 657 of WS protein clearly brings down PCNA. In a systematic attempt to identify the segment of the WS protein responsible for this interaction (Fig. 2C), further analyses were performed with fusion proteins containing different segments of the WS protein. Table I gives a list of all the fusion constructs used in this study. The summary of the data shown in Fig. 2C indicates that PCNA binds to a region of the WS protein containing amino acids 168 to 246.
GST-WS fusion protein. RPA is known to increase the activity of the WS helicase in vitro (27). However, the signal was very weak (data not shown). The result with topoisomerase I was particularly interesting as the yeast Wrn orthologue (Sgs1) can interact with topoisomerases (11). To demonstrate that there was indeed an interaction between the WRN protein and topoisomerase I, immunoprecipitation experiments were performed on the human colon carcinoma SW480 cell line with an antibody against the human WS protein. As shown in Fig. 4, the antibody could immunoprecipitate the WRN protein. This interaction was blocked by a WS peptide. The same blot was probed with an antibody against PCNA to confirm the mouse result and topoisomerase I. As expected, PCNA was detected in the immunoprecipitate. More importantly, the topoisomerase I enzyme was also detected in the immunoprecipitate. These results show an interaction between the WRN protein and topoisomerase I, another component of the DNA replication fork. Antibodies against PARP, the 70-kDa RPA, and DNA polymerase δ failed to reveal these proteins in the immunoprecipitate. It is possible that the interaction of the WS protein with other components of the DNA replication fork might have been disrupted during cell lysis. In addition, the antibody used for immunoprecipitations may recognize and mask epitopes on the WS peptide involved in protein/protein interaction. The possibility of weak or transient interactions between the WS protein and other nuclear proteins with or without DNA damage is still under investigation.

**DISCUSSION**

The human WRN gene codes for a protein containing a 3′-5′ exonuclease (28) and a 3′-5′ DNA helicase domain (29). Considering the high homology between human and mouse homologues (30), we established homozygous mutant embryonic stem cells by deleting part of the helicase domain to assess the cellular role of this helicase (17). We noticed that murine mutant ES cells are more sensitive to both types I and II topoisomerase inhibitors than are wild type ES cells. Similar results were obtained with human WS cell lines (31, 32). Because both of these topoisomerases are involved in DNA replication, it is possible that the WS protein acts in concert with these enzymes as part of a replication structure. Indeed, we have seen that the WS protein, but not the mutant protein, co-purifies with the murine 17 S multiprotein replication complex through a number of purification steps (Fig. 1). These results indicate that the WS protein interacts with components of this replication complex. In addition, affinity chromatography experiments with GST-WS fusion proteins, in vitro pull down experiments, and co-immunoprecipitation experiments with a polyclonal mouse and human anti-WS protein antibodies have indicated that WS protein associates with PCNA protein and topoisomerase I. These results strengthen the hypothesis that WS protein may function at the replication fork. The lack of mouse WS mutant protein in the DNA replication fraction in our experiments may be because of the internal deletion created in the helicase domain which could affect the three-dimensional structure of the protein and its subsequent interaction with any protein complex.

It is clear that the interaction of the WS protein with PCNA requires a relatively short stretch of amino acids (168 to 246) in the N-terminal portion of the WS protein. These amino acids form part of the potential exonuclease domain and contain a DQWKLLRFDVK motif (important residues in bold). The latter is known to be critical for the interaction of several proteins of the replication complex with the interconnecting loop domain of PCNA (black box in Fig. 3C) (33, 34). Our results also indicate that the C-terminal region of PCNA is important for protein/protein interaction as described previously (35). Such data raise the possibility that PCNA increases the activity of the potential 3′-5′ exonuclease domain. Additionally, PCNA could increase the activity of both the exonuclease and helicase domains of the protein toward specific tetrahedral DNA structures (36). In any event, the interaction between PCNA and the WS protein is particularly interesting because PCNA is a potential communication point between a variety of important cellular processes, including cell cycle control, DNA replication, DNA recombination, and repair (34). This raises the interesting possibility that PCNA is a bridging molecule between the WS protein and different components of the replication complex.

A possible function of the WS protein in mammalian cells might be to monitor recombination repair of double-strand breaks at the site of DNA replication. Such an action would be similar to the function of RecQ helicase in *E. coli* (37). Thorough analyses of the involvement of the WS helicase in recombinational repair at replication forks are required to test these possibilities.

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