A Novel Protein Interacts with the Werner’s Syndrome Gene Product Physically and Functionally*

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Werner’s syndrome (WS) is a rare autosomal recessive disorder characterized by premature aging. The gene responsible for WS encodes a protein homologous to Escherichia coli RecQ. Here we describe a novel Werner helicase interacting protein (WHIP), which interacts with the N-terminal portion of Werner protein (WRN), containing the exonuclease domain. WHIP, which shows homology to replication factor C family proteins, is conserved from E. coli to human. Ectopically expressed WHIP and WRN co-localized in granular structures in the nucleus. The functional relationship between WHIP and WRN was indicated by genetic analysis of yeast cells. Disruptants of the SGS1 gene of Saccharomyces cerevisiae, which is the WRN homologue in yeast, show an accelerated aging phenotype and high sensitivity to methyl methanesulfonate as compared with wild-type cells. Disruption of the yeast WHIP (yWHIP) gene in wild-type cells and sgs1 disruptants resulted in slightly accelerated aging and enhancement of the premature aging phenotype of sgs1 disruptants, respectively. In contrast, disruption of the yWHIP gene partially alleviated the sensitivity to methyl methanesulfonate of sgs1 disruptants.

Werner’s syndrome (WS) is a rare autosomal recessive disorder characterized by premature aging and an early onset of age-related diseases including arteriosclerosis, malignant neoplasms, melituria, and cataract (1). Somatic cells derived from WS patients show chromosome instability, a shorter life span in vitro, and accelerated telomere shortening (2, 3). WS cells have subtle defects in DNA replication, resulting in a reduced frequency of firing of replication origins (4). In addition, a large number of reports have shown that many cellular events including DNA repair, transcription, and apoptosis are affected in WS cells (5–7). The gene responsible for WS encodes a protein (WRN) that is a member of the RecQ family of DNA helicases (8). Most of the WS mutations that have been identified are nonsense or frameshift mutations, resulting in the truncation of WRN (9, 10). The clinical features and cellular phenotypes of most WS patients seem to be due to an absolute lack of WRN in the nucleus because the nuclear localization signal of WRN resides in its C-terminal end (11).

The RecQ family includes Escherichia coli RecQ, S. cerevisiae Sgs1, Shizosaccharomyces pombe Rqh1, and five human RecQ helicases, namely DNA helicase Q1/RecQL (RecQL1), WRN, Bloom’s syndrome gene product (BLM), Rhusmund-Thomson’s syndrome gene product (RecQL4), and RecQL5 (12–19). Rhusmund-Thomson’s syndrome also shows some features of the premature aging phenotype, and Bloom’s syndrome is characterized by a predisposition to various malignant neoplasms. In S. cerevisiae, mutations in the SGS1 gene caused premature aging and hyper-recombination phenotypes (20, 21). The sgs1 mutants showed higher sensitivity to MMS and hydroxyurea (22–25, 37). Thus, sgs1 mutants exhibit some of the phenotypes of WS.

WRN has been shown to have DNA helicase and exonuclease activity (26–29). Recent studies (30–32) have revealed that WRN interacts with replication protein A, PCNA, DNA topoisomerase I, and DNA polymerase δ, indicating the involvement of WRN in some aspects of DNA replication. WRN also interacts with the p53 and Ku 70/86 heterodimer, suggesting that WRN is involved in apoptosis and the repair of DNA double strand breaks (7, 33–35). Despite these observations, it is not clear how the dysfunction of WRN is related to the observed phenotypes of WS cells. To obtain further insight into the process in which WRN is involved, we performed a two-hybrid screening using mouse WRN (mWRN) as bait and identified three interacting proteins: Ubc9, SUMO-1 (small ubiquitin-related modifier-1), and a novel protein, WHIP (Werner Helicase Interacting Protein), which is conserved from E. coli to human (36). Here we report that mWRN physically interacts with mWHIP, and the yeast homologue of WRN, Sgs1, genetically interacts with yWHIP.

**EXPERIMENTAL PROCEDURES**

**Two-hybrid Assay**—The yeast strains and plasmids for two-hybrid screening were described previously (36).

**Cloning of Mouse and Human WHIP cDNA**—We cloned partial
mWHIP cDNA lacking the 5' region by two-hybrid screening. To obtain the 5' region of mWHIP, we performed nested PCR using a Cap-site cDNA library of mouse testis (Nippon Gene) as a template, appropriate primers, and the Advantage GC2-PCR kit (CLONTECH). Based on the 5' sequence of mWHIP obtained by nested PCR, full-length mWHIP cDNA was amplified by PCR reaction (RT-PCR) using the Advantage GC2-PCR kit and appropriate primers containing BglII and BamHI sites on total RNA from testes of C57BL/6 mice and cloned into the pGEM-T-Easy vector (Promega).

Construction of Plasmids—The bait plasmid pGBT9-mWRN, plasmids containing various truncated mouse WRN cDNA, pGBT9-mouse BLM, pGBT9-mouse RecQL1α and pGBT9-mouse RecQL1β, and mammalian expression plasmid pFLAG-mWRN were prepared as described previously (36). For in vitro translation, full-length WRN cDNA was amplified by PCR using appropriate primers containing a SalI site, BamHI cDNA in-frame at the SalI and subcloned into a bacterial expression vector encoding MBP-fused mWHIP (pMAL-mWHIP) and the mammalian expression vectors encoding N-terminal HA-tagged mWHIP (pHA-mWHIP) and N-terminal GFP-tagged mWHIP (pEGFP-mWHIP) were constructed by inserting the SalI end of mWHIP cDNA-in-frame at the BamHI site of pMAL-c2 (New England Biolabs), pCMV-Tag1b (CLONTECH), pEGFP-C1 (CLONTECH), and pGFP-C1 (CLONTECH) deletion mutant (SacI/SalI). The bacterial expression vector encoding MBP-fused mWHIP (pMAL-mWHIP) and the mammalian expression vectors encoding N-terminal HA-tagged mWHIP (pHA-mWHIP) and N-terminal GFP-tagged mWHIP (pEGFP-mWHIP) were constructed by inserting the mWHIP cDNA-in-frame at the BamHI site of pMAL-c2, pCMV-Tag1b, pEGFP-C1, and pGFP-C1 deletion mutant (SacI/SalI) into appropriate restriction sites of pMAL-mWHIP, pHA-mWHIP, and pEGFP-mWHIP, respectively.

Northern Blot Analysis—The expression of hWHIP and WRN mRNA was studied using multiple tissue Northern blots (CLONTECH). The filters were hybridized with [α-32P]dCTP-labeled hWHIP and hWHIP cDNA fragments, respectively, at 42 °C overnight in a 5× SSPE buffer containing 50% formamide, 2× SDS, 10× Denhardt's solution, and 100 μg/ml depurinated salmon sperm DNA. The washing was done under highly stringent conditions: three washes with 2× SSC, 0.1% SDS at room temperature and one with 0.2× SSC, 0.1% SDS for 30 min at 65 °C. The filters were analyzed using a BAS 1500 system (Fuji Film).

Expression of WRN and WHIP—Human 293 EBNA cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were grown to 70% confluence in 10-cm dishes, transfected with plasmid DNA using LipofectAMINE (Life Technologies, Inc.), and incubated for 48 h.

Immunoprecipitation and Western Blot Analysis—Transfected and nontransfected 293 EBNA cells were used to detect the interaction of exogenous and endogenous proteins, respectively. The cells were washed once with phosphate-buffered saline, lysed with 0.5% Triton buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% Triton X-100, 50 mM NaCl, 3 mM MgCl2, 300 mM sucrose) for 5 min. After being rinsed three times with PBS, the cells in each well were overlaid with blocking buffer (0.1% skim milk, 0.5% Na2SO4) for 3 h at 37 °C, and the blocking buffer was removed. The cells were incubated with anti-FLAG M2 monoclonal antibody (Eastman Kodak Co.) in PBS containing 1% bovine serum albumin for 12 h at 4 °C. After three washes with PBS, cells were treated for 3 h at 37 °C with Texas red-conjugated goat anti-mouse IgG (Vector Laboratories, Inc.) and washed five times with PBS. Samples were mounted in Permafluor (Lipshaw-Immunon, Inc.) and analyzed with a Bio-Rad MRC-1024 confocal microscope.

Yeast Strains and Plasmids—Yeast strains used in this study were derived from MR966 (MATa ura3–52 leu2–3, 112 trp-289 his1–7). The PCR product was used to transform MR966, and the sgs1 strain and transformants were selected in YPAD medium containing 200 μg/ml Geneticin. The oligonucleotides used were as follows: oligo 1, 5'-ATCCGGTTGCTTGTGGGAAAAGCCGTGTTTGGTGTCAAAACGCTACCGTGAGCTGCG3′-3′; oligo 2, 5′-GAATTGTGACGGGCAGATCGGCTGAAAGATGAGAAGACCGCTTGAGCATCGGATATTCCAGCCTG3′-5′; oligo 3, 5′-CTAGACTCGTGAACACCCCGAAGGAATAGTACGGCGGCAATGCGAACCCTTGGACCATCGGATATTCCAGCCTG3′-5′. The antisera were confirmed to cross-react with WHIP and to be able to immunoprecipitate both mWHIP expressed in E. coli and endogenously expressed mWHIP.

RESULTS AND DISCUSSION

To gain an insight into the cellular processes in which WRN is involved, we tried to identify proteins that interact with WRN by yeast two-hybrid screening using cDNA encoding the mouse WRN as bait. We identified three proteins: a novel protein, which we designated as WHIP, and Ubc9 and SUMO-1, which are involved in the post-replication repair protein, RAD18. To identify proteins that interact with WHIP, we tried to identify proteins that interact with WHIP by yeast two-hybrid screening using cDNA encoding the mouse WHIP. To identify proteins that interact with WHIP, we performed nested PCR using a Cap-site cDNA library of mouse testis (Nippon Gene) as a template, appropriate primers, and the Advantage GC2-PCR kit (CLONTECH). Based on the 5' sequence of WRN obtained by nested PCR, full-length WRN cDNA was amplified by PCR reaction (RT-PCR) using the Advantage GC2-PCR kit and appropriate primers containing BglII and BamHI sites on total RNA from testes of C57BL/6 mice and cloned into a bacterial expression vector encoding MBP-fused mWHIP (pMAL-mWHIP) and the mammalian expression vectors encoding N-terminal HA-tagged mWHIP (pHA-mWHIP) and N-terminal GFP-tagged mWHIP (pEGFP-mWHIP) were constructed by inserting the mWHIP cDNA-in-frame at the BamHI site of pMAL-c2, pCMV-Tag1b, pEGFP-C1, and pGFP-C1 deletion mutant (SacI/SalI) into appropriate restriction sites of pMAL-mWHIP, pHA-mWHIP, and pEGFP-mWHIP, respectively.

Immunofluorescence—The 293 EBNA cells were grown on poly-L-lysine coated 8-chamber culture slides and transfected with plasmid DNA by lipofection. Cells cultured for 24 h after transfection were fixed three times with PBS, fixed with 4% paraformaldehyde in PBS containing 2% sucrose for 10 min, and then permeabilized with Triton buffer (20 mM HEPEs, pH 7.4, 0.5% Triton X-100, 50 mM NaCl, 3 mM MgCl2, 300 mM sucrose) for 5 min. After being rinsed three times with PBS, the cells in each well were overlaid with blocking buffer (0.1% citrate buffer, pH 6.0, skim milk, 0.5% Na2SO4) for 3 h at 37 °C, and the blocking buffer was removed. The cells were incubated with anti-FLAG M2 monoclonal antibody (Eastman Kodak Co.) in PBS containing 1% bovine serum albumin for 12 h at 4 °C. After three washes with PBS, cells were treated for 3 h at 37 °C with Texas red-conjugated goat anti-mouse IgG (Vector Laboratories, Inc.) and washed five times with PBS. Samples were mounted in Permafluor (Lipshaw-Immunon, Inc.) and analyzed with a Bio-Rad MRC-1024 confocal microscope.

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tated with an anti-FLAG antibody. The immunoprecipitants were analyzed by Western blotting using the anti-FLAG antibody and an anti-HA antibody, revealing co-precipitation of mWRN and mWHIP (Fig. 2A). In addition, the direct association between mWRN and mWHIP was confirmed by a pull-down assay for in vitro translated mWRN using MBP-mWHIP (Fig. 2B). To address the interaction between endogenous WRN and WHIP in the cell, we generated anti-WHIP antisera and performed immunoprecipitation using the antisera. As shown in Fig. 2C, endogenous WRN was co-immunoprecipitated with WHIP. We next determined the region of WRN where WHIP binds by using the two-hybrid system. Deletion mutants of mWRN were transfected into yeast cells, and β-galactosidase activity was assayed. Positive results were obtained with constructs encoding polypeptides containing the N-terminal portion of mWRN (1–271 aa) including the exonuclease domain (78–219 aa) but not with the construct encoding the polypeptide corresponding to the region where Ubc9 binds (272–514 aa) (36) (Fig. 2D).

In addition to WRN, there are four recQ homologues in human cells; RECQL1, BLM, RECQL4, and RECQL5. We have previously shown that mUb9 interacts with both mWRN and mBLM but not with mRECQL1 isozymes (36). Thus, we examined whether mWHIP interacts with RecQ family proteins.
other than WRN. As shown in Fig. 3A, mWHIP did not interact with either isomer of mRECQL1 or mBLM. In this context, it is interesting that exogenously expressed mWHIP co-localized with exogenously expressed mWRN in granular structures in the nucleus (Fig. 3B).

Numerous studies have shown that many cellular events including telomere maintenance and DNA replication, DNA repair, transcription, and apoptosis are affected in WS cells. Recently, the focus-forming activity 1, which was found as a factor for recruiting RPA to the pre-replicative foci in a cell-free system, was identified as the Xenopus leavis homologue for WRN (41). In addition, it has been reported that WRN interacts with PCNA, DNA topoisomerase I, and DNA polymerase δ, suggesting that WRN plays some role in DNA replication (30–32). Thus, it is quite interesting that WHIP has motifs similar to those in replication factor C. To examine the functional relationship between WHIP and WRN, we took advantage of yeast genetics, because WHIP is conserved from yeast to human.

In budding yeast, a sole recQ homologue was identified as SGS1 (13). An original mutant allele of SGS1 was identified as a suppressor of the slow growth phenotype of top3 mutants. Deletion mutants of the SGS1 gene showed pleiotropic phenotypes including premature aging of mother cells, poor sporulation, a reduction in the fidelity of chromosome segregation during mitosis and meiosis, and a mitotic hyper-recombination phenotype (13, 20–22, 37, 42, 43). In addition, the sgs1 mutants were shown to be hypersensitive to hydroxyurea and MMS (22–25, 37). Cells derived from WS patients show chromosome aberrations that were not observed in control cells (36). A, B, C, D, and E indicate the exonuclease domain, repeat region, acidic region, helicase domain, and nuclear localization signal, respectively. a.a., amino acid position.

**Fig. 2.** WHIP binds the 1–272 aa region of WRN. A, co-immunoprecipitation of WRN and WHIP. The 293 EBNA cells transfected with pFLAG-mWRN (or pFLAG) and pHA-mWHIP (or pha) were harvested 48 h after transfection. Cells were lysed, and FLAG-mWRN was immunoprecipitated with an anti-FLAG antibody. Whole cell extracts (WCEs) and the immunoprecipitates (IPs) were subjected to 7% SDS-polyacrylamide gel electrophoresis. mWRN and mWHIP were visualized by Western blotting using anti-FLAG and anti-HA antibodies, respectively. The minus symbol indicates transfection of the control plasmid. IB, immunoblot. B, in vitro binding of WHIP to WRN. [35S]methionine-labeled mWRN was incubated with MBP or MBP-mWHIP resins, and proteins bound to the resins were developed by 7% SDS-PAGE and subjected to autoradiography. C, co-immunoprecipitation of endogenous WRN and WHIP. Immunoprecipitations were carried out using anti-WHIP antisera from whole cell extract (WCE) of 293 EBNA cells. WCE and immunoprecipitates were subjected to Western blot analysis. The Western blotting was done with an anti-WRN monoclonal antibody and anti-WHIP antisera. D, binding of WHIP to the 1–271 aa region of WRN in two-hybrid assay. The plasmid pGBT9-mWRN-F or plasmids encoding variously truncated mWRN cDNA were co-transfected with pGAD-mWHIP or control plasmid into the Y190 yeast strain. β-Galactosidase activity was assayed as described previously (36). A, B, C, D, and E indicate the exonuclease domain, repeat region, acidic region, helicase domain, and nuclear localization signal, respectively. a.a., amino acid position.
to possess DNA-dependent ATPase and single-stranded DNA annealing activity (47). Thus, Sgs1 and yWHIP catalyze opposite reactions, the unwinding of double-stranded DNA and annealing of single-stranded DNA, respectively. This fact may help to explain the alleviation of the MMS sensitivity of sgs1 disruptants by disruption of the yWHIP gene.

In conclusion, the physical interaction in mammalian cells and the genetic interaction in budding yeast between WHIP and WRN (Sgs1) indicate a functional link between WHIP and WRN that might be conserved from yeast to human.

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