p53 Modulates RPA-Dependent and RPA-Independent WRN Helicase Activity

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Abstract

Werner syndrome is a hereditary disorder characterized by the early onset of age-related symptoms, including cancer. The absence of a p53-WRN helicase interaction may disrupt the signal to direct S-phase cells into apoptosis for programmed cell death and contribute to the pronounced genomic instability and cancer predisposition in Werner syndrome cells. Results from communoprecipitation studies indicate that WRN is associated with replication protein A (RPA) and p53 in vitro before and after treatment with the replication inhibitor hydroxyurea or γ-irradiation that introduces DNA strand breaks. Analysis of the protein interactions among purified recombinant WRN, RPA, and p53 proteins indicate that all three protein pairs bind with similar affinity in the low nanomolar range. In vitro studies show that p53 inhibits RPA-stimulated WRN helicase activity on an 849-bp M13 partial duplex substrate. p53 also inhibited WRN unwinding of a short (19-bp) forked duplex substrate in the absence of RPA. WRN unwinding of the forked duplex substrate was specific, because helicase inhibition mediated by p53 was retained in the presence of excess competitor DNA and was significantly reduced or absent in helicase reactions catalyzed by a WRN helicase domain fragment lacking the p53 binding site or the human RECQ1 DNA helicase, respectively. p53 effectively inhibited WRN helicase activity on model DNA substrate intermediates of replication/repair, a 5’ ssDNA flap structure and a synthetic replication fork. Regulation of WRN helicase activity by p53 is likely to play an important role in genomic integrity surveillance, a vital function in the prevention of tumor progression. (Cancer Res 2005; 65(4): 1223-33)

Introduction

Werner syndrome is an autosomal recessive disorder that displays symptoms of premature aging, including an elevated incidence of neoplastic cancers, particularly sarcomas (1–3). Werner syndrome cells grown in culture display marked chromosome translocation, rearrangements, and deletions. Mutations in the Werner syndrome (WRN) gene are found in patients exhibiting the clinical symptoms of Werner syndrome (4). The WRN gene encodes a protein that harbors both DNA helicase (5) and exonuclease (6, 7) activities. The precise molecular role(s) of the WRN protein is not known but presumably relates to DNA metabolic pathways that influence genome integrity as suggested by the catalytic activities of the WRN protein and its interactions with nuclear proteins implicated in DNA replication, repair, and recombination (8).

The tumor suppressor gene p53 plays a critical role in the DNA damage response pathway to activate checkpoint control in mammalian cells. Loss of p53 function results in genomic instability, a key feature of carcinogenesis (reviewed in refs. 9–11). Key components of checkpoint control mediated by p53 include arrest of cell cycle progression, inhibition of DNA replication, and activation of DNA repair. A DNA damage signaling pathway leads to elevated p53, which up-regulates transcription of several genes, including the cyclin-dependent kinase inhibitor p21 (12, 13). In addition to growth arrest, p53 mediates damage-induced apoptosis in certain cell types (14). Induction of apoptosis by p53 is thought to be important for the removal of cells from the population that are unable to repair the damage.

Several recent findings suggest that p53 and WRN proteins function together to maintain genomic stability: (a) p53-mediated apoptosis is attenuated in Werner syndrome cells (15); (b) overexpression of WRN results in enhanced p53-dependent transcriptional activity (16); (c) Sp1-mediated transcription of the WRN gene is modulated by p53 (17); and (d) WRN−/− knockout mice display accelerated mortality in a p53-null background (18). In support of a molecular interaction between WRN and p53, a physical interaction between the proteins has been reported (15, 16). Further work has shown p53 can modulate WRN exonuclease activity (19) and Holliday junction unwinding (20).

Recently, several physical and functional interactions between helicase domain-containing proteins and p53 have been discovered. p53 binds to the human transcription factor IIH subunits XPB and XPD and inhibits their helicase activities (21, 22). p53 also interacts with BLM (23, 24), the Cockayne syndrome group B protein (22), and SV40 large T antigen (25, 26). p53 is also able to inhibit the helicase activity catalyzed by the large T antigen (27, 28) as well as its replication function (29). In addition to helicases, p53 physically and functionally interacts with the ssDNA binding protein replication protein A (RPA; refs. 30, 31). p53 inhibits ssDNA binding by RPA and blocks SV40 viral replication via its interaction with RPA (30).

Our recent work showed that RPA physically interacts with WRN (32), BLM (33), and RECQ1 (34) helicases and stimulates their respective helicase activities. A specific interaction between human RecQ helicases and RPA is further supported by the absolute requirement for RPA in the WRN-, BLM-, or RECQ1-catalyzed unwinding of long DNA duplexes (32–34). Because RPA has been shown to have roles in DNA replication, recombination, and repair, human RecQ helicases are likely to function with the ssDNA binding protein during one or more of these processes.
The p53-RPA, RPA-WRN, and p53-WRN connections suggest that the phenotypes of cancer and genomic instability in Werner syndrome may possibly involve p53 modulation of WRN catalytic functions affected by RPA. To investigate this possibility, we have directly examined the effect of p53 on WRN helicase activity in the presence or absence of RPA. Our results show that p53 can modulate RPA-dependent WRN helicase activity on a long 849-bp DNA substrate. On forked DNA structures with a short (19-bp) duplex region, p53 directly inhibited WRN helicase activity in a specific manner. Regulation of DNA unwinding activity by p53 is relevant to the important roles of RecQ helicases in the maintenance of genome stability.

Materials and Methods

Cells and Treatments. The normal human diploid fibroblasts (MRC-5) or Werner syndrome fibroblasts (AG11395) were grown in DMEM supplemented with 10% fetal bovine serum at 37°C in 5% CO2 Hydroxyurea (Sigma, St. Louis, MO) was added to the 50% to 80% confluent cultures from a 200 mm flask solution to a final concentration of 2 mmol/L, for 15 hours until harvesting. For treatment with ionizing radiation, cells were washed with PBS and then irradiated at 6 Gy using Gammacell 40, a 137Cs source emitting at a fixed dose rate of 0.82 Gy/min (Nordion International, Ottawa, Ontario, Canada). After ionizing radiation treatment, cells were cultured in DMEM for 2 hours before harvesting.

Coimmunoprecipitation Experiments. MRC-5 and AG11395 cells (1 x 10^7) were collected by low-speed centrifugation, washed in cold PBS, and lysed in whole cell lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% NP40, 2 mmol/L Na3VO4, 10 mmol/L NaF] containing protease inhibitors (Roche, Indianapolis, IN). For coimmunoprecipitation experiments, whole cell lysate (1 mg protein) was incubated with either rabbit polyclonal anti-WRN antibody (H-300, 1:60 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit IgG (Santa Cruz Biotechnology) either in the presence or in the absence of ethidium bromide (10 μg/mL) for 4 to 6 hours at 4°C. The mixture was subsequently tumbled with 40 μL protein G agarose (Roche) at 4°C overnight. Beads were washed thrice with the whole cell lysis buffer supplemented with 0.1% Tween 20. Protein complexes were eluted by boiling in SDS sample buffer and resolved on 10% polyacrylamide Tris-glycine SDS gels followed by transfer to polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ). The membranes were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween 20 and probed for WRN, RPA, and p53 using antibodies against WRN (mouse monoclonal at 1:250, BD Transduction, San Diego, CA), 70- and 32-kDa subunits of RPA (Ab-1 and Ab-3 at 1:20, Oncogene Research, San Diego, CA), and p53 (Ab-6 at 1:1,000, Oncogene Research), respectively, followed by horseradish peroxidase conjugated to horseradish peroxidase (Amersham Biosciences). Proteins on immunoblots were detected using Enhanced Chilumiluminescence Plus (Amersham Biosciences).

Proteins. Baculovirus constructs for recombinant hexa-histidine-tagged, full-length wild-type or exonuclease-defective WRN protein (WRN-E84A, designated X-WRN) were kindly provided by Dr. Matthew Gray (University of Washington, Seattle WA) and Dr. Judith Campisi (Lawrence Berkeley National Laboratory, Berkeley, CA), respectively. Amplified baculovirus was used to infect Sf9 insect cells for WRN protein overexpression, and WRN was purified to apparent homogeneity as described elsewhere (35). Recombinant glutathione S-transferase-WRN800-996 fusion protein was overexpressed in Escherichia coli and purified using glutathione beads (Amersham Biosciences) as described previously (35). Recombinant human RecQ1 was overexpressed in insect cells using a baculovirus encoding recombinant RecQ1 kindly provided by Dr. Alessandro Vindigni (International Center for Genetic Engineering and Biotechnology, Trieste, Italy) and purified as described elsewhere (34). Human RPA containing all three subunits (RPA70, RPA32, and RPA44) was purified as described previously (36). p53 was purified as described previously (22). T4 polynucleotide kinase was obtained from New England Biolabs (Beverly, MA). T7 Sequenase version 2.0 was purchased from U.S. Biochemical (Cleveland, OH).

Nucleotides and DNA. M13mp18 single-stranded circular DNA was from New England Biolabs. The two oligonucleotides used for the 34-bp telomeric forked duplex DNA substrate were 5'-TTTTTTTTTTTTTTTTTTAGGTTAGGTTAGGTTAGGCTACATC-3' and 5'-GTAGTGATGATCATGCC-TAACCCCTAAGCTTACACCCCTAATTTTTTTTTTTTTT-3'. Yeast tRNA was from Boehringer Mannheim (Indianapolis, IN). [γ-32P]ATP was from Perkin Elmer (Wellesley, MA).

Duplex DNA Helicase Substrates. The 849-bp M13mp18 partial duplex substrate was constructed as described previously (32). Brieilly, the gel-purified 849-bp duplex DNA fragment from the HaeIII digest of M13mp18 RF was treated with calf intestinal phosphatase to remove 5' phosphates and subsequently labeled at their 5' ends using T4 polynucleotide kinase and [γ-32P]ATP. The complementary fragments were then annealed to M13mp18 ssDNA circle. M13 partial duplex DNA substrates were purified by gel filtration column chromatography using A-5M resin (Bio-Rad, Hercules, CA). The 34-bp forked duplex DNA substrate was prepared as described previously (37). The 19-bp forked duplex, 26-nucleotide 5' flap with either the upstream or the downstream primer 5' 32P labeled, and synthetic replication fork DNA substrates were constructed as described previously (38).

Helicase Assays. Helicase assay reaction mixtures (20 μL) for assays using the 849-bp M13 partial duplex substrate (0.125 mmol/L) contained 40 mmol/L Tris (pH 7.4), 4 mmol/L MgCl2, 5 mmol/L DTT, 2 mmol/L ATP, 0.5 mg/ml tRNA, and the indicated concentration of DNA protein. For WRN or WRN500-996 helicase assays using the oligonucleotide-based duplex substrates, reaction mixtures (20 μL) contained 30 mmol/L HEPES (pH 7.5), 40 mmol/L KCl, 8 mmol/L MgCl2, 2 mmol/L ATP 100 mg/ml bovine serum albumin (BSA), 5% glycerol, and DNA substrate concentrations indicated in the figure legends. For those WRN helicase reactions containing RPA or wild-type p53, the concentrations are indicated in the figure legends. Reaction mixtures were preincubated with RPA and the indicated concentration of p53 protein on ice for 4 minutes and subsequently initiated by the addition of WRN protein. WRN helicase reactions were incubated at either 24°C for 60 minutes for the assays using the M13 partial duplex substrates or 37°C for 15 minutes using the oligonucleotide-based duplex substrates. For RecQ1 helicase assays, reaction mixtures (20 μL) contained 20 mmol/L Tris-HCl (pH 7.4), 8 mmol/L DTT, 5 mmol/L MgCl2, 5 mmol/L ATP, 10 mmol/L KCl, 4% (w/v) sucrose, 80 μg/mL BSA, and 0.5 mmol/L forked duplex DNA substrate. RecQ1 helicase reactions were incubated at 37°C for 15 minutes. Reactions were terminated by the addition of 10 μL of 50 mmol/L EDTA-40% glycerol-0.9% SDS-0.1% bromphenol blue-0.1% xylene cyanol. The products of helicase reactions were resolved on 6 or 12% polyacrylamide gels. Radiolabeled DNA species or polyacrylamide gels were visualized using a PhosphorImager and quantitated using the ImageQuant software (Molecular Dynamics, Piscataway, NJ). The percentage helicase substrate unwound was calculated as described previously (38).

For ssDNA competitor experiments, p53 (42 mmol/L) was preincubated with the indicated concentrations (0-50 mmol/L) of "unlabeled" ssDNA (25-mer oligonucleotide) in standard helicase reaction buffer for unwinding assays with oligonucleotide-based substrates (see above) containing 2 mmol/L ATP for 3 minutes at 24°C. Forked duplex substrate (10 fmol radiolabeled 19 bp, final concentration 0.5 mmol/L) and WRN (final concentration 3 mmol/L) was simultaneously added to the reaction mixture after the 3-minute preincubation and incubated subsequently for 7 minutes at 37°C. Reactions were then quenched and resolved on native polyacrylamide gels as described above. Typically, 70% to 85% of the forked duplex substrate was unwound in reactions lacking the DNA competitor molecule. Unwinding (% control) is expressed relative to the control reactions lacking the competitor DNA.

Strand Displacement Synthesis Reactions. WRN and p53 were preincubated on ice for 3 minutes in the presence of 10 fmol of a 26-nucleotide 5' flap substrate (upstream primer 5' 32P labeled) in 30 mmol/L HEPES (pH 7.5), 40 mmol/L KCl, 8 mmol/L MgCl2, 2 mmol/L...
ATP, 100 μg/mL BSA, 0.5 μmol/L deoxynucleotide triphosphates, and 5% glycerol. T7 Sequenase (U.S. Biochemical, 25 mU) was added and the 20 μL reactions were incubated at 37°C for 5 minutes and terminated with the addition of 10 μL formamide loading buffer. Reactions were resolved on 20% denaturing gels containing 7 mol/L urea. Radiolabeled DNA species in polyacrylamide gels were visualized using a PhosphorImager.

**Electrophoretic Mobility Shift Assays.** Electrophoretic mobility shift assays were similar to those described previously (20). Briefly, reaction mixtures (20 μL) contained 30 μmol/L HEPES (pH 7.5), 40 mmol/L KCl, 8 mmol/L MgCl₂, 100 ng/μL BSA, 5% glycerol, 0.5 mmol/L of the 19-bp forked duplex DNA substrate, and the indicated p53 concentrations. Reactions were incubated at 24°C for 20 minutes followed by fixation for 10 minutes at 37°C in the presence of 0.25% glutaraldehyde. Products were resolved by 5% nondenaturing PAGE at 4°C for 2.5 hours and visualized using a PhosphorImager.

**ELISA Detection of Protein Interaction among WRN, RPA, and p53.** Purified WRN, p53 or RPA proteins were diluted to a concentration of 1 ng/μL in carbonate buffer [0.016 mol/L Na₂CO₃, 0.034 mol/L NaHCO₃ (pH 9.6)], coated to appropriate wells of a 96-well microtiter plate (50 μL/well), and allowed to incubate overnight at 4°C. Control wells were incubated with carbonate buffer and incubated overnight at 4°C. Wells were aspirated and blocked for 2 hours at 30°C with blocking buffer (3% BSA in PBS with 0.5% Tween 20). Wells were aspirated and washed again; all washing steps were done with the blocking buffer. Wells were then coated with serial dilutions of WRN, RPA, or p53 in 30 mmol/L HEPES (pH 7.5), 40 mmol/L KCl, 8 mmol/L MgCl₂, 100 ng/μL BSA, and 5% glycerol starting with 36 nmol/L protein. For the ethidium bromide treatment, 10 μg/mL ethidium bromide were included in the incubation with WRN, RPA, or p53 during the binding step. Following incubation for 1 hour at 30°C, wells were aspirated, washed five times, and allowed to incubate for 1 hour at 30°C with anti-WRN (1:500, rabbit polyclonal, Novus Biologicals, Littleton, CO), anti-RPA (1:100 mouse monoclonal, Oncogene Research), or anti-p53 antibody (1:1,000, Oncogene Research) diluted in blocking buffer. Following three washings, horseradish peroxidase–conjugated anti-rabbit (1:5,000, Santa Cruz Biotechnology) or anti-mouse (1:5,000, Amersham Biosciences) antibody was added to the wells and incubated for 30 minutes at 30°C. After washing five times, WRN and p53 were detected using OPD substrate (Sigma). The reaction was terminated after 3 minutes with 3 N H₂SO₄ and absorbance readings were taken at 490 nm. The absorbance was corrected for the background signal in the presence of BSA.

**ELISA Data Analysis.** The fraction of the immobilized WRN, RPA, or p53 bound to the microtiter well that was specifically bound by WRN or p53 protein was determined from the ELISA assays. A Hill plot was used to analyze the data as described previously (33).

### Results

It was shown previously that the COOH terminus of p53 (15, 16) physically interacts with the COOH terminus of WRN. In support of the direct physical interaction, it was shown that p53 modulates the exonuclease activity of WRN protein (19). No direct effect of p53 on WRN ATPase or helicase activity on a 28-bp M13 partial duplex substrate was observed (19). However, p53 has been shown to inhibit both WRN and BLM helicase activities on a Holliday junction (20). The physical interaction between p53 and WRN or RPA (30, 31, 39) suggested to us that WRN helicase activity on longer duplex DNA substrates might be affected by the presence of p53 in the reaction, because RPA is an essential ancillary factor for WRN unwinding of DNA duplexes at least 257 bp (32). In addition, we have examined the effect of p53 on WRN helicase activity on biologically relevant DNA structures that are proposed intermediates during cellular DNA transactions. The ability of p53 to directly modulate the unwinding reaction of a human helicase would alter the ability of the enzyme to function in a pathway of DNA metabolism and may have potential consequences for genome integrity.

**Effect of p53 on WRN Helicase Activity on M13 Partial Duplex Substrates.** To determine if p53 exerts a direct effect on WRN unwinding of standard B-form duplex DNA, we preincubated WRN helicase (92 nmol/L monomer) with increasing amounts of p53 (0, 47, 94, and 184 nmol/L monomer) before incubation with a M13 28-bp partial duplex DNA substrate and found that p53 did not stimulate or inhibit WRN-catalyzed unwinding of the M13 partial duplex (19). Moreover, p53 (47-184 nmol/L) did not stimulate WRN helicase activity on the 28-bp partial duplex substrate with an amount of WRN protein (26 nmol/L monomer) that unwinds only 10% of the duplex DNA substrate (19).

We reported previously a physical and functional interaction between WRN and RPA (32). The presence of RPA stimulates WRN helicase to unwind DNA duplexes as long as 849 bp (32). p53 has been shown to associate with RPA both in vivo and in vitro (30, 31, 39). In addition, human p53 can inhibit SV40 viral DNA replication by its association with RPA (30). The nature of the replication function of RPA that is inhibited by p53 is not known. We hypothesized that p53 may inhibit RPA-dependent WRN helicase activity. To address this issue, an 849-bp M13 partial duplex DNA substrate was tested for WRN helicase activity in the presence of RPA and increasing concentrations of p53 (0, 47, 94, and 188 nmol/L p53 monomer). Inhibition of WRN helicase activity was detected at a p53 concentration of 47 nmol/L (Fig. 1, lane 3) and more evident at p53 concentrations of 94 and 188 nmol/L (Fig. 1, lanes 4 and 5). At 188 nmol/L p53, WRN unwinding of the 849-bp substrate was inhibited ~70% compared with WRN helicase reactions lacking p53. Addition of up to 4-fold more RPA (final concentration 372 nmol/L) in the WRN helicase reaction on the 849-bp duplex substrate did not alleviate the p53 inhibition (data not shown), suggesting that the mechanism of p53 inhibition involves more than simply p53 sequestration of RPA. Inhibition of WRN helicase activity was dependent on the duplex length of the M13 substrate, because less inhibition was observed for 341- or 100-bp partial duplex substrates, and p53 failed to inhibit WRN helicase activity on a 69-bp M13 partial duplex substrate in the presence of RPA (data not shown).

**Effect of p53 on Telomere Duplex Fork Unwinding by WRN.** The p53 inhibition of RPA-dependent WRN helicase activity on long M13 partial duplex substrates characterized by vast regions of ssDNA (~7,000 nucleotides) raised the question if p53 might retain its ability to modulate WRN-catalyzed DNA unwinding on a more...
physiologic substrate with reduced ssDNA character. Biochemical characterization of the DNA substrate specificity of the WRN helicase showed that a forked DNA duplex with noncomplementary 3’ and 5’ ssDNA tails is a preferred structure for DNA unwinding by WRN (38). Forked DNA substrates are important DNA structural intermediates in a variety of DNA metabolic pathways, including replication, repair, and recombination. To investigate if p53 modulates WRN helicase activity on B-form DNA of a more physiologically relevant substrate, we tested the effect of p53 on RPA-dependent WRN helicase activity on a 34-bp duplex with 15-nucleotide 3’ and 5’ ssDNA tails. Because WRN may have a direct role in telomere metabolism, we chose a forked duplex of human telomeric repeat sequences (TTAGGG). In order for this telomeric duplex substrate to be unwound by WRN, RPA is required in the helicase reaction (37). We elected to test the effect of p53 on RPA-stimulated helicase activity of a mutant WRN protein (X-WRN) devoid of exonuclease activity due to a missense mutation in the active site of the exonuclease domain. The use of purified X-WRN recombinant protein enabled us to examine the effect of p53 on RPA-stimulated WRN helicase on the 34-bp forked duplex substrate without having to take into account the inhibitory effect of p53 on WRN 3’ to 5’ exonuclease activity that can degrade from the 3’ termini of blunt ends of forked duplex molecules that the helicase does not efficiently unwind (37).

As shown in Fig. 2, lane 3, the 34-bp forked duplex was not detectably unwound by X-WRN (1.5 nmol/L monomer). However, in the presence of RPA (9.8 nmol/L), WRN helicase unwound 62% of the 34-bp duplex (Fig. 2, lane 4). The presence of p53 in the WRN-RPA helicase reaction resulted in the inhibition of WRN helicase activity as evidenced by the disappearance of the released strands with the concomitant appearance of intact duplex DNA substrate resolved on the native polyacrylamide gel (Fig. 2, lanes 5-9). Inhibition of WRN helicase activity on the 34-bp forked duplex substrate was p53 concentration dependent. At the highest p53 concentration tested (123 nmol/L), WRN helicase activity was inhibited by ~50% of the control (no p53) unwinding reaction. These results show that p53 effectively inhibits WRN helicase activity that requires RPA to unwind forked DNA duplexes as short as 34 bp. These results also show that the inhibition of RPA-dependent WRN helicase activity exerted by p53 does not require long ssDNA regions on the helicase substrate because the forked duplex with 15-nucleotide 3’ and 5’ ssDNA tails is effectively inhibited by p53. We conclude that RPA-dependent WRN-catalyzed unwinding of the telomeric duplex fork is significantly reduced by p53.

**p53 Inhibition of RPA-Independent WRN Helicase Activity.**

To determine if p53 had the ability to inhibit RPA-independent WRN helicase activity, we tested a forked DNA substrate with a short (19-bp) duplex region. WRN (3 nmol/L) was able to unwind 78% of the forked duplex substrate in the absence of RPA (Fig. 3A, lane 2). In the presence of 11 nmol/L p53, WRN helicase activity on the forked duplex decreased by 30% compared with control reactions lacking p53 (Fig. 3A, lane 3, and B). At the highest concentration of p53 (42 nmol/L), there was a 90% decrease in WRN helicase activity on the forked duplex (Fig. 3A, lane 4, and B). These results show that p53 inhibits WRN helicase activity on a short (19-bp) forked duplex that WRN unwinds efficiently in the absence of its auxiliary factor RPA.

![Figure 2](image2.png)

**Figure 2.** p53 inhibits RPA-dependent WRN helicase activity on a forked DNA duplex of human telomeric sequence. An exonuclease defective WRN mutant protein, X-WRN (1.5 nmol/L), was incubated with the 34-bp forked duplex (0.9 nmol/L) in the absence or presence of RPA (9.8 nmol/L heterotrimer) and the indicated concentrations of p53 monomer for 15 minutes at 37°C as described in Materials and Methods. Products were resolved on native 12% polyacrylamide gels. Lane 1, heat-denatured substrate control.

![Figure 3](image3.png)

**Figure 3.** p53 inhibits WRN helicase activity on a short (19-bp) forked duplex substrate. A. WRN protein (3 nmol/L) was incubated with the 19-bp forked duplex (0.5 nmol/L) in the presence of the indicated concentration of p53 for 15 minutes at 37°C as described in Materials and Methods. Products were resolved on native 12% polyacrylamide gels. Heat-denatured DNA substrate control (▲). B. Quantitation of % control helicase activity from experiments as conducted in A.
An interaction between p53 and the forked duplex DNA substrate might contribute to the inhibition of WRN helicase activity on this substrate because p53 is known to bind to DNA structures, including Holliday junctions (40). Using a gel mobility shift assay to measure DNA binding, we observed that indeed p53 was able to bind the forked duplex substrate used for the helicase studies in a specific manner dependent on the p53 concentration (Fig. 4).

The ability of p53 to bind the forked 19-bp duplex raised the possibility that the p53 inhibition of WRN helicase activity may in part be mediated by a p53-DNA interaction as opposed to a p53-WRN protein interaction. To address this issue, we examined the effect of p53 on WRN unwinding of the same forked duplex substrate but with the additional presence of a 25-nucleotide ssDNA competitor, because p53 binds to ssDNA and the forked duplex substrate contains 25- and 26-nucleotide ssDNA arms. As shown in Fig. 5, p53 retained its ability to inhibit WRN unwinding of the forked duplex substrate (0.5 nmol/L) in the presence of competitor ssDNA (12.5-50 nmol/L), suggesting that p53 is inhibiting WRN helicase activity by a mechanism other than p53 simply binding to the ssDNA arms of the forked duplex substrate and preventing WRN from either binding or efficiently initiating DNA unwinding of the forked duplex substrate. Similar competition experiments with cold forked duplex DNA (41) or blunt duplex DNA (42) inhibited WRN helicase activity in the absence of p53, which prevented us from assessing the ability of p53 to inhibit WRN helicase activity in the presence of these DNA competitor molecules.

Effect of p53 on Unwinding Catalyzed by WRN Helicase Domain Fragment or Human RECQ1 Helicase. To better understand the nature of the p53 inhibition of WRN-catalyzed unwinding of the forked duplex, we compared the effect of p53 on DNA unwinding catalyzed by full-length WRN with a recombinant glutathione S-transferase-WRN helicase domain fragment (glutathione S-transferase-WRN(500-946)) that lacks the COOH-terminal p53 interaction domain (WRN residues 1013-1432). p53 (21 nmol/L) inhibited the unwinding catalyzed by the WRN helicase domain fragment to some extent (29% inhibition) but not as greatly as that observed for full-length WRN (55% inhibition; Fig. 6A and B), suggesting that the p53 protein interaction with WRN significantly contributes to the helicase inhibition.

To evaluate the specificity of WRN helicase inhibition by p53, we tested its effect on another human RecQ DNA helicase, RECQ1. As shown in Fig. 6C and D, RECQ1 DNA helicase activity was completely resistant to a concentration of p53 (42 nmol/L) that under the same reaction conditions nearly eliminated WRN unwinding of the 19-bp forked duplex substrate. These results indicate that p53 inhibition of WRN helicase activity is specific and not a general effect exerted on any human RecQ helicase.

p53 Inhibition of WRN Helicase Activity on DNA Replication and Repair Intermediates. Our previous studies showed that WRN helicase efficiently unwinds two important DNA intermediates of replication/repair, a 5' ssDNA flap and a synthetic replication fork (38). WRN was able to translocate on the lagging strand of the synthetic replication fork to unwind duplex ahead of the fork. For the 5' flap structure, WRN specifically displaced the 5' flap oligonucleotide, suggesting a role of WRN in Okazaki fragment processing or DNA repair. To address a potential role of p53 in modulation of WRN helicase activity on these replication/repair intermediates, we tested for its effect on WRN-catalyzed unwinding of the 5' flap structure (Fig. 7A) or synthetic replication fork structures (Fig. 7C). The results, shown quantitatively in Fig. 7B and D, show that p53 effectively inhibited WRN helicase activity on both DNA substrates. The ability of p53 to block WRN unwinding of the replication fork structure indicates that the p53 inhibition does not require any preexisting ssDNA in the helicase substrate to prevent WRN from unwinding the DNA structure.

Because p53 inhibited WRN helicase activity on the 5' flap structure, we were interested in the possibility that it would modulate the extent of strand displacement by DNA polymerase synthesis that is stimulated by WRN helicase activity. To address this experimentally, we did primer extension assays using a 5' flap substrate in which the upstream primer was 5' 32P-labeled. Strand displacement DNA synthesis was stimulated by WRN helicase activity as evidenced by the increased production of longer 32P-labeled single strands resolved on denaturing polyacrylamide gels (compare lane 2 with lane 3 in Fig. 7E). Enhancement of strand displacement DNA synthesis was dependent on WRN helicase activity, because a helicase-dead ATPase mutant of WRN did not stimulate DNA synthesis under conditions in which the wild-type WRN enzyme did as observed previously for the effect of WRN on strand displacement synthesis by polymerase (42). When p53 was included in the DNA synthesis reaction mixture with WRN (Fig. 7E, lane 4), the synthesized DNA products were shorter and more closely resembled those from reactions lacking WRN (Fig. 7E, lane 2). In control reactions, p53 did not have an effect on DNA synthesis catalyzed in the absence of WRN (Fig. 7E, lane 5). Under the reaction conditions for strand displacement DNA synthesis reaction mixtures with cold forked duplex DNA (41) or blunt duplex DNA (42) inhibited WRN helicase activity in the absence of p53. 

4 Unpublished data. 
5 Unpublished data.
In the immunoprecipitation reactions (data not shown), no detectable signal was obtained for WRN, RPA, and p53, used for the coimmunoprecipitation (2 mmol/L hydroxyurea (lane 5) and RPA was also evident in cells that had been exposed to WRN in untreated cells (lane 4)). The association of WRN with p53 and RPA was observed in the presence of ethidium bromide from the normal cell extracts (Fig. 9B), suggesting that a DNA bridge was not responsible for their coimmunoprecipitation. These results show that endogenous WRN-p53 and WRN-RPA complexes exist in human cells during normal DNA metabolism and persist during replication arrest or DNA processing in response to DNA damage.

**Physical Analysis of the Protein Interactions among WRN, p53, and RPA.** Collectively, the results from the p53-WRN helicase studies suggested that direct protein interactions among WRN, RPA, and p53 are likely to be important in the inhibition of WRN helicase activity on the various DNA substrates. To characterize the physical protein interactions, we did ELISA studies to determine the relative binding affinities of the protein pairs (WRN-RPA, WRN-p53, and RPA-p53). Purified recombinant p53 protein (0-36 nmol/L) was incubated in the presence of 3% BSA with WRN or RPA that had been immobilized on microtiter wells. Bound p53 was detected using anti-p53 antibodies. The specificity of this interaction was shown by very low absorbance values (0.089 ± 0.012 /ₐ₉₀) for wells that had been precoated with BSA compared with the intense signal obtained with WRN or RPA (Fig. 8A). The colorimetric signal from the WRN-p53 or RPA-p53 interaction was both dose dependent and saturable. The data analyzed by Scatchard binding theory using a Hill plot were linear, indicating a single site on p53 for binding to either WRN or RPA. The apparent dissociation constants (Kₐ) for p53-WRN and p53-RPA were 1.81 and 2.22 nmol/L, respectively (Table 1), indicating that p53 binds to WRN with similar affinity to that observed with the p53-RPA interaction.

To validate the results, we modified the ELISA procedure by coating either p53 or RPA on the microtiter wells and incubating with WRN in the presence of 3% BSA in the helicase reaction buffer. Bound WRN was detected using anti-WRN antibodies. As observed in the previous set of ELISA assays, the colorimetric signal was both dose dependent and saturable (Fig. 8B). The apparent dissociation constants for WRN-RPA and WRN-p53 were 1.40 and 1.23 nmol/L, respectively (Table 1), indicating that WRN bound to RPA with similar affinity to that observed with the WRN-p53 interaction.

The colorimetric signals from the WRN-p53 (Fig. 8C), WRN-RPA (Fig. 8C), and RPA-p53 (Fig. 8D) interactions were resistant to the presence of ethidium bromide during binding, indicating that a contaminating DNA bridge was not responsible for the positive signal.

**In vivo Interaction of WRN with RPA and p53 in Untreated and DNA Damaged Cells.** To explore the possibility that endogenous WRN, RPA, and p53 are associated with each other in vivo, we did coimmunoprecipitation experiments using whole cell lysates prepared from human diploid fibroblasts that were either untreated or treated with the replication inhibitor hydroxyurea or ionizing radiation that introduces DNA strand breaks. Polyclonal antibody against WRN protein was used to precipitate endogenous WRN and associated p53 or RPA. As shown in Fig. 9A, both p53 and RPA were coimmunoprecipitated with WRN in untreated cells (lane 4). The association of WRN with p53 and RPA was also evident in cells that had been exposed to 2 mmol/L hydroxyurea (lane 5) or 6 Gy ionizing radiation (lane 6). Input for the various lysates represented 10% of the total protein used for the coimmunoprecipitation (lanes 1-3). In control experiments, no detectable signal was obtained for WRN, RPA, or p53 when antibody was omitted or normal rabbit IgG was used in the immunoprecipitation reactions (data not shown). The specificity of the WRN antibody was shown by the result that p53 or p53 was not precipitated by the anti-WRN antibody from the WRN-/- cell extracts in which WRN was absent (Fig. 9B). In addition, both p53 and RPA were coimmunoprecipitated with WRN by the anti-WRN antibody in the presence of ethidium bromide from the normal cell extracts (Fig. 9B), suggesting that a DNA bridge was not responsible for their coimmunoprecipitation. These results show that endogenous WRN-p53 and WRN-RPA complexes exist in human cells during normal DNA metabolism and persist during replication arrest or DNA processing in response to DNA damage.

**Discussion**

In this report, we have shown that p53 modulates both RPA-dependent and RPA-independent WRN helicase activity on B-form duplex DNA substrates. To gain some insight to the nature and specificity of WRN helicase inhibition by p53, we have focused on further characterizing the effect of p53 on short oligonucleotide-based substrates that have only minimal ssDNA character. From these analyses, we conclude that p53 modulates WRN helicase activity in a specific manner because it does not impair the DNA unwinding reaction on a forked duplex catalyzed by a related DNA synthesis, p53 inhibited WRN unwinding of the 5' flap oligonucleotide. These results indicate that p53 modulates strand displacement DNA synthesis by inhibiting WRN helicase activity on the 5' flap structure.

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6 Unpublished data.
human RecQ helicase, RECQ1. A significant component of the WRN helicase inhibition by p53 is likely to be mediated by a direct protein interaction because the presence of exogenous DNA competitor did not alleviate the inhibitory effect of p53 on WRN unwinding. In support of this, a WRN helicase domain fragment lacking the p53 binding site was not inhibited to as great an extent as the full-length WRN protein, suggesting that p53 binding to the DNA substrate was not the major contributor to WRN helicase inhibition by p53. In addition to these functional assays, physical analyses of the WRN-p53 interaction show that the two proteins interact with a high affinity, and this interaction is not mediated by DNA.

The ability of p53 to deter WRN unwinding of the 5' flap and synthetic replication fork structures suggests that p53 may modulate WRN helicase activity on DNA intermediates that arise during DNA replication and/or repair. A role of WRN in processing 5’ DNA flap structures is suggested by our biochemical and genetic results that show a specific interaction between WRN and FEN-1 (35, 43, 44), a structure-specific nuclease implicated in trimming of 5’ flaps created during strand displacement synthesis in either lagging strand replication or DNA repair processes, such as base excision repair (reviewed in ref. 45). The 5’ flap substrate is efficiently unwound by WRN (38), and modulation of this activity by p53 may be important to prevent the creation of long 5’ flaps.

Figure 6. Effect of p53 on unwinding catalyzed by WRN helicase domain fragment or human RECQ1 helicase. A, glutathione-S-transferase-WRN<sub>500-946</sub> (9.4 nmol/L) or WRN (1.5 nmol/L) was incubated with the 19-bp forked duplex (0.5 nmol/L) in the presence of p53 (21 nmol/L) for 15 minutes under standard reaction conditions at 37°C as described in Materials and Methods. C, RECQ1 (10 nmol/L) or WRN (0.8 nmol/L) was incubated with the 19-bp forked duplex (0.5 nmol/L) in the presence of p53 (42 nmol/L) for 15 minutes under RECQ1 helicase reaction conditions at 37°C as described in Materials and Methods. Products were resolved on native 12% polyacrylamide gels. Heat-denatured DNA substrate control (▲). B and D, quantitation of percent helicase activity from experiments as conducted in A and C, respectively. Columns, average of at least three independent experiments; bars, SD.
Figure 7. p53 modulates WRN helicase activity on synthetic DNA intermediates of replication and repair. WRN (3 or 6.8 nmol/L, respectively) was incubated with the 5' flap DNA substrate (0.5 nmol/L; A) or the synthetic replication fork substrate (0.5 nmol/L; C) in the presence of p53 (42 nmol/L) for 15 minutes under standard reaction conditions at 37°C as described in Materials and Methods. Products were resolved on native 12% polyacrylamide gels. Heat-denatured DNA substrate control (E). B and D, quantitation of percent helicase activity from experiments as conducted in A and C, respectively. Columns, average of at least three independent experiments; bars, SD. E, effect of p53 on strand displacement DNA synthesis stimulated by WRN helicase activity. As described in Materials and Methods, T7 Sequenase was incubated with 26-nucleotide 5' flap substrate (10 fmol, upstream primer 5'32P labeled) for 5 minutes at 37°C in the absence of WRN and p53 (lane 2), in the presence of 4.8 nmol/L WRN (lane 3), in the presence of 4.8 nmol/L WRN and 70 nmol/L p53 (lane 4), or in the presence of 70 nmol/L p53 only (lane 5). Products were resolved on denaturing polyacrylamide gels. Representative phosphoimage of a gel.
resistant to subsequent processing by FEN-1. By its ability to regulate WRN helicase activity, p53 would serve to insure proper maturation of the newly synthesized lagging strand. In this context, we have shown that p53 modulates the ability of WRN helicase to facilitate strand displacement by DNA polymerase synthesis. p53 may be involved in replication- or repair-related processes by its ability to modulate WRN helicase activity on key DNA metabolic intermediates.

The inhibition of RPA-dependent WRN helicase activity on long DNA duplex substrates is of interest because p53 did not have an effect on WRN helicase activity on shorter partial duplex substrates characterized by the same M13 ssDNA backbone. The fact that p53, WRN, and RPA all bind ssDNA and each other makes it difficult to elucidate the mechanism of inhibition. Nonetheless, the results presented also show that WRN can block RPA-dependent unwinding of a more physiologically relevant substrate, the telomeric forked duplex, suggesting that the involvement of p53 in DNA transactions catalyzed by WRN may be biologically significant.

| Table 1. Physical analysis of WRN, RPA, and p53 protein interactions |
|--------------------------|------------------|
| Protein interaction      | $K_d$ (nmol/L)   |
| (protein titrated-protein immobilized) |        |
| WRN-RPA                  | 1.395            |
| WRN-p53                  | 1.233            |
| p53-WRN                  | 1.808            |
| p53-RPA                  | 2.219            |

NOTE: $K_d$ determinations were based on the average of three independent ELISA experiments as shown in Fig. 8.

Information pertaining to the physical interaction sites among WRN, RPA, and p53 should prove useful to understanding the mechanism of p53 inhibition of WRN-catalyzed unwinding. WRN protein can bind to p53 via its COOH-terminal 419 residues, and an interaction domain for WRN has been mapped to the last 100 amino acids of p53 (full-length 393 amino acids; refs. 15, 16). Both an amino region [residues 1-73 (31) and 2-117 (30)] and a COOH region [residues 289-393 (30)] of p53 interact with the 70-kDa subunit of the RPA heterotrimer. Residues 1 to 221 and 411 to 492 of RPA70 interact with p53 (39). Thus, it is possible that p53 can bind to both WRN and RPA simultaneously; therefore, we cannot rule out that a dual protein interaction may be responsible for p53 inhibition of RPA-stimulated WRN helicase activity. WRN binds to the 70-kDa subunit of RPA, and the WRN binding motif is located within amino acids 100 to 300 and overlaps with the ssDNA binding domain (amino acids 150-450) of RPA70 (46). Because p53 interacts with the NH2-terminal half (residues 1-221) of the 70-kDa RPA subunit (39), it is possible that binding of p53 to the 70-kDa subunit prevents the interaction of RPA with WRN during the unwinding reaction. Alternatively, it was shown previously that p53 associates with RPA and prevents RPA from binding ssDNA (30). A more detailed understanding of the mechanism for RPA stimulation of WRN helicase activity will be helpful to delineate the precise mechanism of p53 inhibition of RPA-dependent WRN helicase activity.

The inhibition of WRN helicase activity by p53 is likely to have biological consequences. p53 is able to inhibit SV40 viral replication (29) and nuclear DNA replication in a transcription-free DNA replication extract from *Xenopus* eggs (47), suggesting that p53 may bind directly to proteins of the replication complex and interfere with DNA replication. RPA is required for replication of chromosomal DNA and displays specific functional and physical interactions with the WRN and BLM DNA helicases.
WRN may function during replication as suggested by the extended S phase (48) and a reduced frequency of initiation sites in Werner syndrome cells (49, 50). Genomic instability in Werner syndrome cells, characterized by extensive deletions and chromosomal rearrangements, may arise due to basic defects in a replication-associated process that involves both RPA and p53.

The interaction of p53 with WRN protein and/or the WRN-RPA complex may be critical to deter entry into S phase or to direct S-phase cells into apoptosis. Recent studies suggest that RPA binding by p53 is less important for growth suppression than the transactivation and transrepression functions of p53 (51). Thus, the p53-RPA interaction may be critical for other cellular processes, such as DNA repair or apoptosis. The attenuation of p53-mediated apoptosis in Werner syndrome cells (15) may possibly be explained by the absence of a p53-WRN direct interaction that could serve as a signal for programmed cell death. p53-mediated arrest of WRN-catalyzed unwinding during the initiation or elongation phases of DNA replication may be responsible for the apoptotic signal. Protein synthesis is not required for p53-induced apoptosis, suggesting that p53 directly targets downstream members, such as WRN, in the apoptotic pathway (52). Our findings suggest that the WRN as well as BLM enzymes (24) may be downstream targets of p53 in a pathway of DNA metabolism regulated by p53. p53 has been proposed to serve as a "molecular governor" of homologous recombination by functionally interacting with BLM and Rad51 during resolution of stalled replication forks, supporting a S-phase-specific and transcriptional activity (16). p53 may exert its effect on WRN via its interaction with RPA. The genomic instability and cancer predisposition observed in Werner syndrome may be related to a defect in p53-mediated apoptosis, as they are required for enhancement of p53-dependent transcriptional activity (16). p53 may have a role to inhibit strand exchange or replication fork regression that is promoted by Rad51 (53) and possibly facilitated by WRN.

p53 modulation of WRN catalytic function may be an important feature of p53-mediated apoptosis. Defects in this pathway may contribute to the prevalent cancer disposition in Werner syndrome patients. Spillare et al. (15) showed that p53-mediated apoptosis in Werner syndrome fibroblasts could be rescued by expression of wild-type WRN protein. It would be insightful to determine if expression of the COOH-terminal domain of WRN responsible for physical interaction with p53 is sufficient for rescue of the attenuated p53-mediated apoptosis in WS−/− cells. Perhaps, other domains of WRN are also involved in p53-mediated apoptosis as they are required for enhancement of p53-dependent transcriptional activity (16). p53 may exert its effect on WRN via its interaction with RPA. The genomic instability and cancer predisposition observed in Werner syndrome may be related to a defect in p53-mediated apoptosis that is dependent on the helicase function of WRN. Precisely defining the cellular DNA metabolic pathways on which p53 regulates WRN function in vivo is the next challenge.

Acknowledgments

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References


Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22-24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 891; substitute for the last paragraph:

The data in Table 8 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to \( O_2 \) consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if \( M = +0.27 \) and \( L = -0.16 \) and the normal differential is 65 per cent M and 35 per cent L, then

\[
0.65 (+0.27) + 0.35 (-0.16) = +0.12
\]

a figure identical to the observed +0.12 for normal leukocytes.
p53 Modulates RPA-Dependent and RPA-Independent WRN Helicase Activity

Joshua A. Sommers, Sudha Sharma, Kevin M. Doherty, et al.