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Sharma, S ; Sommers, J A ; Gary, R K ; Friedrich-Heineken, E ; Hübscher, U ; Brosh, R M

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The interaction site of Flap Endonuclease-1 with WRN helicase suggests a coordination of WRN and PCNA

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ABSTRACT

Werner and Bloom syndromes are genetic RecQ helicase disorders characterized by genomic instability. Biochemical and genetic data indicate that an important protein interaction of WRN and Bloom syndrome (BLM) helicases is with the structure-specific nuclease Flap Endonuclease 1 (FEN-1), an enzyme that is implicated in the processing of DNA intermediates that arise during cellular DNA replication, repair and recombination. To acquire a better understanding of the interaction of WRN and BLM with FEN-1, we have mapped the FEN-1 binding site on the two RecQ helicases. Both WRN and BLM bind to the extreme C-terminal 18 amino acid tail of FEN-1 that is adjacent to the PCNA binding site of FEN-1. The importance of the WRN/BLM physical interaction with the FEN-1 C-terminal tail was confirmed by functional interaction studies with catalytically active purified recombinant FEN-1 deletion mutant proteins that lack either the WRN/BLM binding site or the PCNA interaction site. The distinct binding sites of WRN and PCNA and their combined effect on FEN-1 nuclease activity suggest that they may coordinately act with FEN-1. WRN was shown to facilitate FEN-1 binding to its preferred double-flap substrate through its protein interaction with the FEN-1 C-terminal binding site. WRN retained its ability to physically bind and stimulate acetylated FEN-1 cleavage activity to the same extent as unacetylated FEN-1. These studies provide new insights to the interaction of WRN and BLM helicases with FEN-1, and how these interactions might

be regulated with the PCNA–FEN-1 interaction during DNA replication and repair.

INTRODUCTION

Werner syndrome (WS) is a rare genetic premature aging disorder characterized by genomic instability (1). The replication (2–4) and recombination (5,6) defects of WS cells, as well as their hypersensitivity to DNA damaging agents (7–10), suggest that WRN processes genomic DNA structures that arise at the elongating or stalled replication fork. Indeed, the WRN protein has multiple DNA metabolic functions that include DNA unwinding dependent on ATP hydrolysis (11,12), 3′–5′ exonuclease activity (13–15) and strand annealing (16). A number of proteins involved in cellular DNA metabolism physically and/or functionally interact with WRN, supporting the notion that WRN participates in multiple pathways by virtue of its intrinsic catalytic activities and protein interactions [for review see (17,18)].

Of the numerous WRN protein interactions reported, we have been particularly interested in the interaction of WRN with Flap Endonuclease 1 (FEN-1) (19), a structure-specific nuclease implicated in DNA replication, repair and recombination [for review see (20)]. Genetic and biochemical evidence implicate FEN-1 in the process of Okazaki fragment processing through its ability to cleave a double-flap DNA substrate that arises during DNA synthesis strand displacement. Yeast studies have also implicated FEN-1 in the maintenance of genomic stability, DNA damage response and stabilization of telomeres. Mouse FEN-1 null blastocysts display proliferation failure and gamma radiation sensitivity (21). FEN-1 haploinsufficiency in mice can lead to tumor progression (22), suggesting that FEN-1, like WRN, serves as a tumor suppressor by its role in genome stability maintenance.

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Evidence for an *in vivo* function of the WRN-FEN-1 interaction in DNA replication was attained using a model yeast-based system for genetic complementation analysis (23). WRN was demonstrated to rescue the cellular phenotypes of a *dna2* mutant defective in a helicase–nuclease that participates with FEN-1 in Okazaki fragment processing. Genetic complementation studies indicated that human WRN rescues *dna2-1* mutant phenotypes of growth, cell cycle arrest and sensitivity to the replication inhibitor hydroxyurea or DNA damaging agent methylmethane sulfonate. Importantly, expression of a conserved non-catalytic domain of WRN that mediates the physical and functional interaction with FEN-1 was sufficient to complement the *dna2-1* mutant phenotypes, suggesting a role of the conserved non-catalytic domain of a RecQ helicase in DNA replication intermediate processing.

In human cells, fluorescence resonance energy transfer (FRET) analyses showed that WRN and FEN-1 form a complex that co-localizes in foci associated with arrested replication forks (24). Biochemical analyses demonstrated that WRN and FEN-1 together process branch-migrating DNA structures associated with the replication fork (24). Molecular and cellular evidence demonstrate that the Bloom syndrome helicase (BLM) also interacts physically with FEN-1 and stimulates the FEN-1 cleavage reaction through a region of the BLM C-terminal domain that shares homology with the FEN-1 interaction domain of WRN (25). The physical and functional interactions of the human RecQ helicases BLM and WRN with FEN-1 are probable to be important for the roles of these proteins in the maintenance of genome stability.

To gain further insight to how the FEN-1 cleavage reaction is stimulated by WRN or BLM, we have performed mapping studies to determine the interaction site on FEN-1. These results indicate that WRN or BLM interacts with a site on FEN-1 that is distinct from its other interacting partner PCNA. FEN-1 can become acetylated in response to DNA damage, causing a marked reduction in its cleavage activity (26). Acetylated FEN-1 was stimulated by WRN, suggesting a potential mechanism for modulating FEN-1 catalyzed DNA cleavage during DNA replication and repair.

MATERIALS AND METHODS

Recombinant proteins

Recombinant His-tagged WRN protein was overexpressed using a baculovirus/*Sf9* insect system and purified as described previously (24). Recombinant His-tagged WRN_{940–1432}, overexpressed in *Escherichia coli*, was purified as published (27). Recombinant His-tagged FEN-1 (wild-type), FEN-1ΔP (residues 337–344 are deleted) or FEN-1ΔC (residues 360–380 are deleted) were overexpressed in *E. coli* and purified as described previously (28). Recombinant PCNA was kindly provided by Dr Mark Kenny (Albert Einstein Cancer Center). Recombinant His-tagged BLM protein was purified as described previously (29). Histone acetyltransferase p300 (p300-HAT) was purchased from Active Motif.

GST–FEN-1–Sepharose affinity pull-down experiments

Details pertaining to the bacterial expression plasmids encoding GST–FEN-1 recombinant protein fragments are found

in (30). GST–FEN-1 fusion proteins were overexpressed in BL21(DE3) pLysS by 1 mM isopropyl-B-D-thiogalactopyranoside induction for 8 h at 23°C. The bacterial cell pellet was lysed by sonication in Lysis buffer [phosphate-buffered saline (PBS), 10% glycerol and 0.4% Triton X-100]. The lysate was clarified by centrifugation at 35 000 r.p.m. (T_i 60 rotor, Beckman) for 1 h at 4°C. Of the resulting supernatant 1 ml was incubated with 100 μl of glutathione S-transferase beads (50% v/v) for 1 h at 4°C. The beads were washed three times with 1 ml Lysis buffer, and split into two aliquots, one for binding experiments and one for determination of background signal in western blot analysis. For binding experiments, protein-bound beads were incubated for 1 h at 4°C with 200 ng of purified recombinant WRN or BLM proteins in 250 μl of Buffer D [50 mM HEPES (pH 7.1), 100 mM KCl and 10% glycerol]. The beads were subsequently washed three times with 500 μl of Buffer D and eluted by boiling treatment in 40 μl of Laemmli buffer [62.5 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate, 25% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue]. Proteins were electrophoresed on 8–16% gradient polyacrylamide SDS gels and transferred onto PVDF membranes. Control membranes were stained with amido black reagent to demonstrate protein loading for samples. Membranes were probed with rabbit polyclonal anti-WRN or anti-BLM antibodies (1:250 and 1:500, respectively) followed by horseradish peroxidase (HRP)-conjugated horse anti-rabbit secondary antibody (1:5000; Santa Cruz Biotech) and ECL-Plus (Amersham Pharmacia).

In vitro acetylation assays

Reactions (30 μl) contained 2 μg of purified recombinant FEN-1, 0.1 μCi [¹⁴C]acetyl coenzyme A (Amersham Pharmacia) and 100 ng p300-HAT in 50 mM Tris–HCl (pH 8.0), 10% (v/v) glycerol, 150 mM NaCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM sodium butyrate. Reactions were incubated at 30°C for 30 min, and used for FEN-1 incision assays (described below) and SDS–PAGE analysis on 10% polyacrylamide gels. Gels were stained with Coomassie and subjected to film autoradiography.

Subnuclear fractionation and immunoprecipitation experiments

HeLa cells were washed with cold PBS, resuspended in Lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% NP-40, 2 mM Na₃VO₄, 10 mM NaF, and 2 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin and 1 mM PMSF] for 30 min on ice and centrifuged at 15 000 g for 20 min at 4°C. The supernatant was recovered as total soluble fraction and the pellet was washed once again with the Lysis buffer and the insoluble chromatin was extracted with 0.5 M NaCl in the Lysis buffer as the high salt fraction. The final chromatin pellet was resuspended in Laemmli buffer and sonicated for 15 s in Microson Ultrasonic Cell Disruptor. Each protein fraction, corresponding to an equivalent cell number, was loaded for 10% SDS–PAGE and analyzed by immunoblotting with indicated antibodies. For the preparation of chromatin fraction used for the immunoprecipitation experiments, sequential subnuclear fractionation from HeLa cells was based on the method of

(31). In brief, cells were resuspended and permeabilized in low salt buffer [10 mM HEPES (pH 7.4), 10 mM KCl and 50 µg/ml digitonin] containing protease inhibitors (2 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml aprotinin and 1 mM PMSF) and 1 mM Na₃VO₄ for 15 min at 4°C. Permeabilized nuclei were recovered by centrifugation at 1000 *g* for 5 min at 4°C followed by two additional washes in the low salt buffer. The nuclei were subsequently resuspended in low salt buffer and incubated with 30 U of RNase-free DNase I (Roche Molecular Biochemicals) for 15 min at 24°C followed by an additional 15 min at 37°C. Proteins from this DNase I-treated nuclei fraction were solubilized by adding Extraction buffer [1% Triton X-100, 50 mM HEPES (pH 7.4), 150 mM NaCl, 30 mM Na₄P₂O₇·10H₂O, 10 mM NaF and 1 mM EDTA] containing protease and phosphatase inhibitors (see above) and incubating for 10 min at 4°C. The supernatant that was collected from centrifugation (20000 *g*, 10 min) was termed the chromatin fraction. Protein (1 mg) from the chromatin fraction was diluted 10-fold in Extraction buffer containing protease inhibitors but lacking Triton X-100, and subsequently incubated with anti-FEN-1 (25) or anti-WRN (H-300, Santa Cruz Biotech.) antibodies for 6 h at 4°C. Protein A–Sepharose (50 µl) (Amersham Pharmacia Biotech) was added, and the resulting mix was rotated at 4°C for overnight. The beads were then washed three times in Tris-buffered saline [50 mM Tris–HCl (pH 8.0) and 150 mM NaCl] containing 0.1% Triton X-100, and the immunoprecipitated proteins were eluted by boiling with Laemmli sample buffer. Samples were electrophoresed on 10% SDS–PAGE, transferred to PVDF membranes, and analyzed by western blot. WRN and FEN-1 were detected using mouse monoclonal anti-WRN (1:250, Novus) and anti-FEN-1 (25) antibodies, respectively.

ELISA studies

Purified recombinant FEN-1 proteins (wild-type FEN-1, FEN-1ΔP, FEN-1ΔC) were diluted to a concentration of 1 ng/ml in Carbonate buffer (0.016 M Na₂CO₃ and 0.034 M NaHCO₃, pH 9.6) and were added to appropriate wells of a 96-well microtiter plate (50 µl/well), which was incubated at 4°C. BSA was used in the coating step for control reactions. The samples were aspirated, and the wells were blocked for 2 h at 30°C with Blocking buffer (PBS, 0.5% Tween 20 and 3% BSA). The procedure was repeated. WRN was diluted in Blocking buffer, and the indicated concentrations were added to the appropriate wells of the ELISA plate (50 µl/well), which was incubated for 1 h at 30°C. For ethidium bromide (EtBr) treatment, 50 µg/ml EtBr was included in the incubation with WRN during the binding step in the corresponding wells. The samples were aspirated, and the wells were washed five times before addition of anti-WRN antibody (H-300; Santa Cruz Biotech.) that was diluted 1:500 in Blocking buffer. Wells were then incubated at 30°C for 1 h. Following three washings, HRP-conjugated anti-rabbit secondary antibody (1:5000) was added to the wells, and the samples were incubated for 30 min at 30°C. After washing five times, any WRN bound to the immobilized FEN-1 was detected using OPD substrate (Sigma). The reaction was terminated after 3 min 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. The fraction of the immobilized FEN-1

bound to the microtiter well that was specifically bound by WRN was determined from the ELISA assays. A Hill plot was used to analyze the data as described previously (32).

Oligonucleotide substrates

PAGE-purified oligonucleotides (Midland Certified Reagent Co., Midland, TX or Lofstrand Labs, Gaithersburg, MD) were used for preparation of DNA substrates. The 26 nt 5' flap, nicked duplex, and double flap with 1 nt 3' noncomplementary tail substrates were prepared as described previously (24,33).

Electrophoretic mobility shift assays

The indicated concentrations of WRN (60, 120, 240 nM) and FEN-1 or FEN-1ΔC (60, 120 nM) were incubated in Binding buffer [50 mM Tris–HCl (pH 8.0), 10 mM NaCl, 5 mM EDTA, 10% glycerol and 50 µg/ml BSA] with 50 fmol double-flap substrate in a total volume of 20 µl at 25°C for 15 min. Protein–DNA complexes were resolved on native 5% polyacrylamide 0.5× TBE gels, and visualized by phosphorimage analysis.

FEN-1 incision assays

Reactions (20 µl) contained 10 fmol of the indicated DNA substrate and the specified amounts of WRN, WRN_{949–1432}, BLM, FEN-1, FEN-1ΔP and/or FEN-1ΔC in 30 mM HEPES (pH 7.6), 5% glycerol, 40 mM KCl, 0.1 mg/ml BSA, and 8 mM MgCl₂. WRN, WRN_{949–1432} or BLM was mixed with the substrate and buffer on ice prior to the addition of FEN-1. Reactions were incubated at 37°C for 15 min and were terminated by the addition of 10 µl of Formamide stop solution [80% formamide (v/v), 0.1% bromophenol blue and 0.1% xylene cyanol], and heated to 95°C for 5 min. Products were resolved on 20% polyacrylamide, 7 M urea denaturing gels. A PhosphorImager was used for detection and ImageQuant software (Molecular Dynamics) was used for quantification of the reaction products. Percent incision was calculated as described previously (19). Cleavage data represent the mean of at least three independent experiments with SDs shown by error bars.

RESULTS

WRN or BLM binding activity resides within a short C-terminal region of FEN-1 adjacent to the PCNA interacting domain

To map the WRN and BLM interaction sites on FEN-1, we tested a series of GST fusion proteins that contain various regions of human FEN-1 for WRN/BLM binding activity using a pull-down affinity bead interaction assay (Figure 1). WRN or BLM binding activity was contained entirely within amino acids 363–380 of FEN-1. All fusion proteins that contained this region bound WRN or BLM, whereas none of the recombinant proteins tested that lacked the complete 18 amino acid sequence displayed binding activity.

It was reported previously that PCNA interacts with FEN-1 by a conserved PCNA binding box motif residing within residues 328–355 of FEN-1 (30). In contrast, FEN-1 residues 363–380 do not interact with PCNA (30). These results and our current mapping data for WRN suggest that the PCNA and

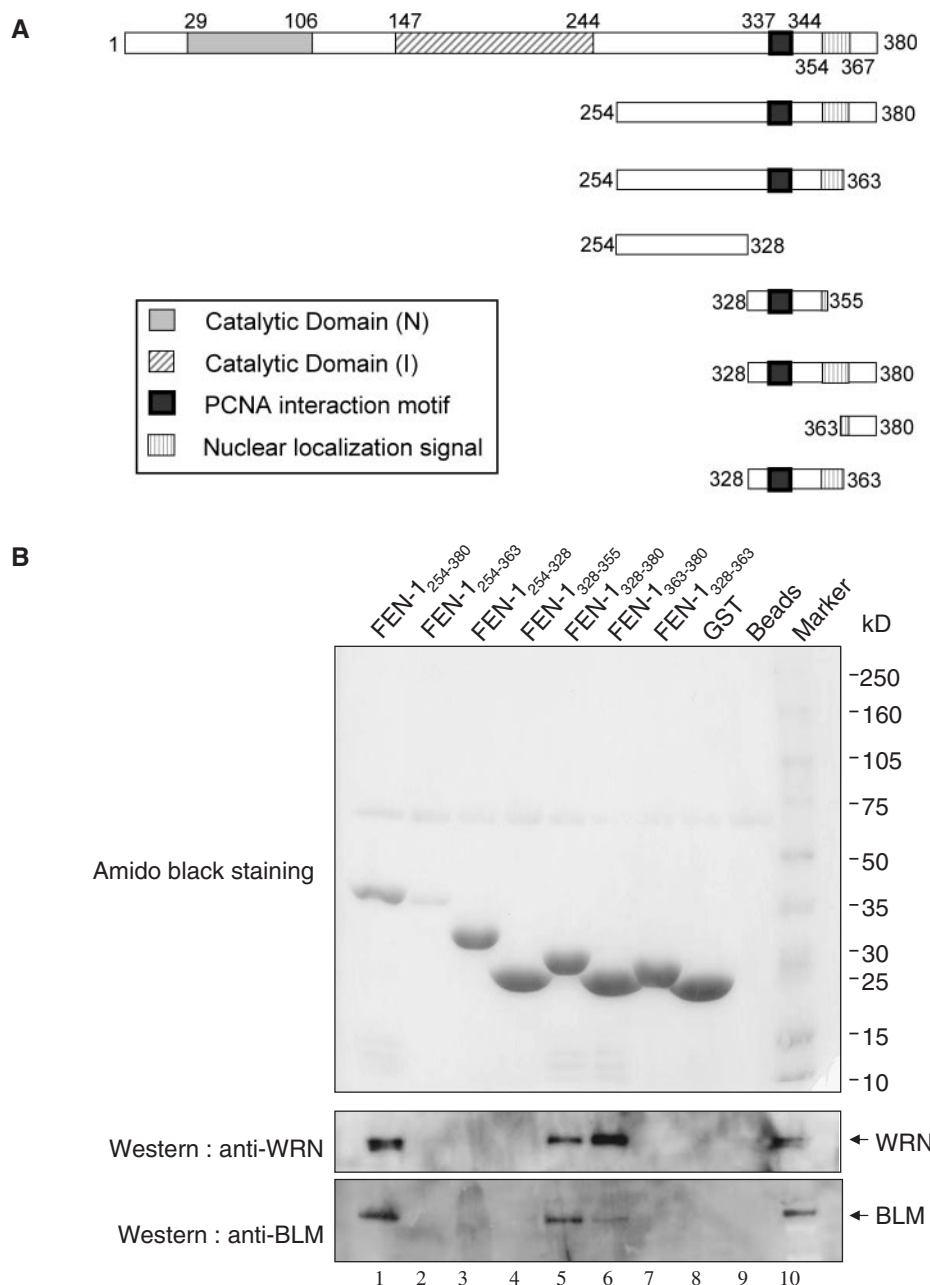


Figure 1. WRN or BLM binding activity of FEN-1 is contained within amino acids 363–380. **(A)** Schematic representation of GST-FEN-1 recombinant fragments used for WRN pull-down experiments. **(B)** Amido black-stained membrane of protein complexes bound to glutathione-agarose beads in pull-down binding assay. Beads were mixed with lysate from bacteria expressing GST fusion proteins containing human FEN-1 amino acids 254–380 (lane 1), 254–363 (lane 2), 254–328 (lane 3), 328–355 (lane 4), 328–380 (lane 5), 363–380 (lane 6), 328–363 (lane 7), GST alone (lane 8) or the agarose beads (lane 9). Purified recombinant WRN or BLM proteins (200 ng) were added to the indicated affinity beads. After washing, protein complexes were eluted and analyzed by SDS-PAGE. Bound WRN (upper panel) or BLM (lower panel) was detected by western blot analysis as described in Materials and Methods. Purified recombinant WRN or BLM protein (50 ng) was used as a marker for western blot (lane 10). In other experiments, greater amounts of the fusion protein GST-FEN-1₂₅₄₋₃₆₃ bound to glutathione beads that were more similar to the other GST fusion proteins did not pull down WRN or BLM (data not shown).

WRN interaction sites on FEN-1 reside adjacent to each other in the C-terminal region of the FEN-1 protein. To address the relative importance of the two C-terminal regions of FEN-1 in mediating the WRN interaction, we tested two deletion mutant forms of FEN-1, one lacking the PCNA binding region (residues 337–344, FEN-1 Δ P) and the other lacking the WRN binding region (residues 360–380, FEN-1 Δ C) for interaction

with WRN by ELISA assay, and compared these results with those obtained for the WRN interaction with full-length FEN-1 (Figure 2). Deletion of residues 360–380 corresponding to the WRN-interacting region of FEN-1 significantly reduced the ability of WRN to bind to the immobilized FEN-1. However, deletion of residues 337–344 corresponding to the PCNA interacting region of FEN-1 did not hamper

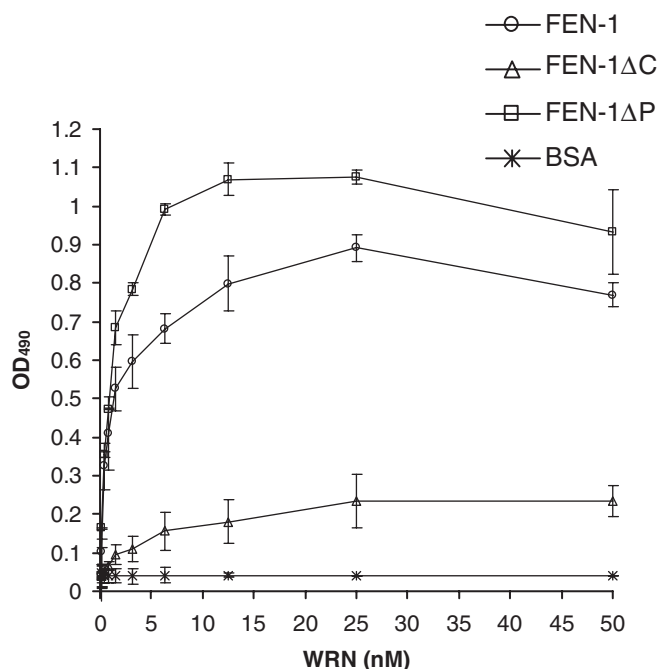


Figure 2. Detection of WRN–FEN-1 complexes by ELISA. Wild-type FEN-1, FEN-1ΔC or FEN-1ΔP was coated onto ELISA plates. Following blocking with 3% BSA, the wells were incubated with increasing concentrations of purified recombinant WRN (0–50 nM) for 1 h at 30°C, and bound WRN was detected by ELISA using a rabbit polyclonal antibody against WRN followed by incubation with secondary HRP-labeled antibodies and OPD substrate. Data points are the mean of three independent experiments performed in duplicate with SDs indicated by error bars.

WRN binding to FEN-1. These results lead us to conclude that WRN physically binds to the last 18 amino acids of the C-terminal tail of FEN-1, and that the adjacent PCNA binding domain of FEN-1 is not required for the physical interaction of WRN with FEN-1.

WRN stimulates the nucleolytic activity of FEN-1 by physically interacting with its 360–380 C-terminal residues

Previously, we had demonstrated that the functional interaction between WRN and FEN-1 whereby WRN stimulates the endonucleolytic and exonucleolytic functions of FEN-1 on 5' flap and nicked duplex DNA substrates is mediated by a non-catalytic C-terminal domain of WRN that physically interacts with FEN-1 (19). These results suggested (but did not prove) that the physical protein interaction between WRN and FEN-1 was required for the functional interaction. However, given the fact that C-terminal fragments of WRN that harbor the RQC or HRDC domains bind DNA (34), it was conceivable that the interaction of the WRN non-catalytic domain with the DNA substrate might be solely responsible for the stimulation of FEN-1 incision activities. To experimentally address the importance of the physical interaction between WRN and FEN-1 for the functional interaction, we examined the ability of WRN to stimulate the cleavage activity of the FEN-1 deletion mutant lacking the WRN-interacting domain (FEN-1ΔC). The FEN-1ΔC recombinant protein was shown to have reduced enzymatic activity; however, the

incision activity of FEN-1 ΔC could be stimulated by PCNA (28). The results from FEN-1 incision assays demonstrate that the cleavage activity of FEN-1ΔC lacking the WRN-interacting region was not stimulated by WRN protein on a 26 nt 5' flap substrate (Figure 3A, lanes 6 and 7, and Figure 3C). In control reactions, WRN stimulated the cleavage activity of full-length FEN-1 by ~7-fold (Figure 3A, lanes 2 and 3, and Figure 3C), consistent with previous results (19,23). We also tested the effect of WRN on the cleavage activity catalyzed by the FEN-1ΔP and found that its cleavage activity was stimulated similar to that observed for the full-length FEN-1 (Figure 3A and C). These results indicate that the WRN-interacting region of FEN-1, but not the PCNA binding domain of FEN-1, is required for WRN to stimulate FEN-1 endonucleolytic cleavage of the 5' flap substrate.

We next tested the ability of a non-catalytic WRN domain fragment (WRN_{949–1432}) that physically interacts with FEN-1 to stimulate the FEN-1ΔC and FEN-1ΔP recombinant proteins. As observed for the full-length WRN protein, WRN_{949–1432} failed to stimulate 5' flap cleavage by FEN-1ΔC (Figure 3B, lanes 6 and 7, and Figure 3C), but retained the ability to stimulate cleavage catalyzed by either full-length FEN-1 (Figure 3B, lanes 2 and 3, and Figure 3C) or the FEN-1ΔP (Figure 3B, lanes 4 and 5, and Figure 3C). Taken together with the data from the FEN-1 incision assays with full-length WRN, the results support those obtained from the physical interaction mapping studies and provide evidence that the direct binding of WRN to FEN-1 is responsible for the functional interaction between the proteins.

In addition to its endonucleolytic activity, FEN-1 can function as an exonuclease on nicked duplex DNA substrates. To address whether or not the WRN-interacting region of FEN-1 is important for the stimulatory effect of WRN on FEN-1 exonuclease activity, we examined incision activity of FEN-1 and its deletion derivatives on a nicked duplex substrate. The results from these studies, shown in Figure 4, demonstrate that the exonuclease activity of FEN-1ΔC was not affected by WRN whereas the presence of WRN stimulated wild-type FEN-1 exonuclease activity on the nicked duplex as expected. Similarly, WRN also retained the ability to stimulate the exonuclease activity of FEN-1ΔP (data not shown). WRN_{949–1432} also stimulated the exonuclease activity of wild-type FEN-1 or FEN-1ΔP on the nicked duplex, but not the exonuclease activity of FEN-1ΔC (data not shown). From these results, we conclude that the WRN-interacting region of FEN-1 is required in order for WRN to stimulate the FEN-1 exonuclease activity on nicked DNA duplex molecules.

Stimulation of FEN-1 cleavage by WRN in the presence of PCNA

The physical and functional mapping results indicate that WRN stimulates FEN-1 cleavage by its binding to the C-terminal tail of FEN-1. Since the WRN interaction site on FEN-1 resides adjacent to, but does not overlap with the PCNA interaction site, we wanted to determine what effect the combination of both WRN and PCNA in the FEN-1 incision reaction might have. To examine the possibility that both WRN and PCNA might retain their respective abilities to stimulate FEN-1 cleavage in the presence of each other, we used limiting amounts of the proteins such that an additive or

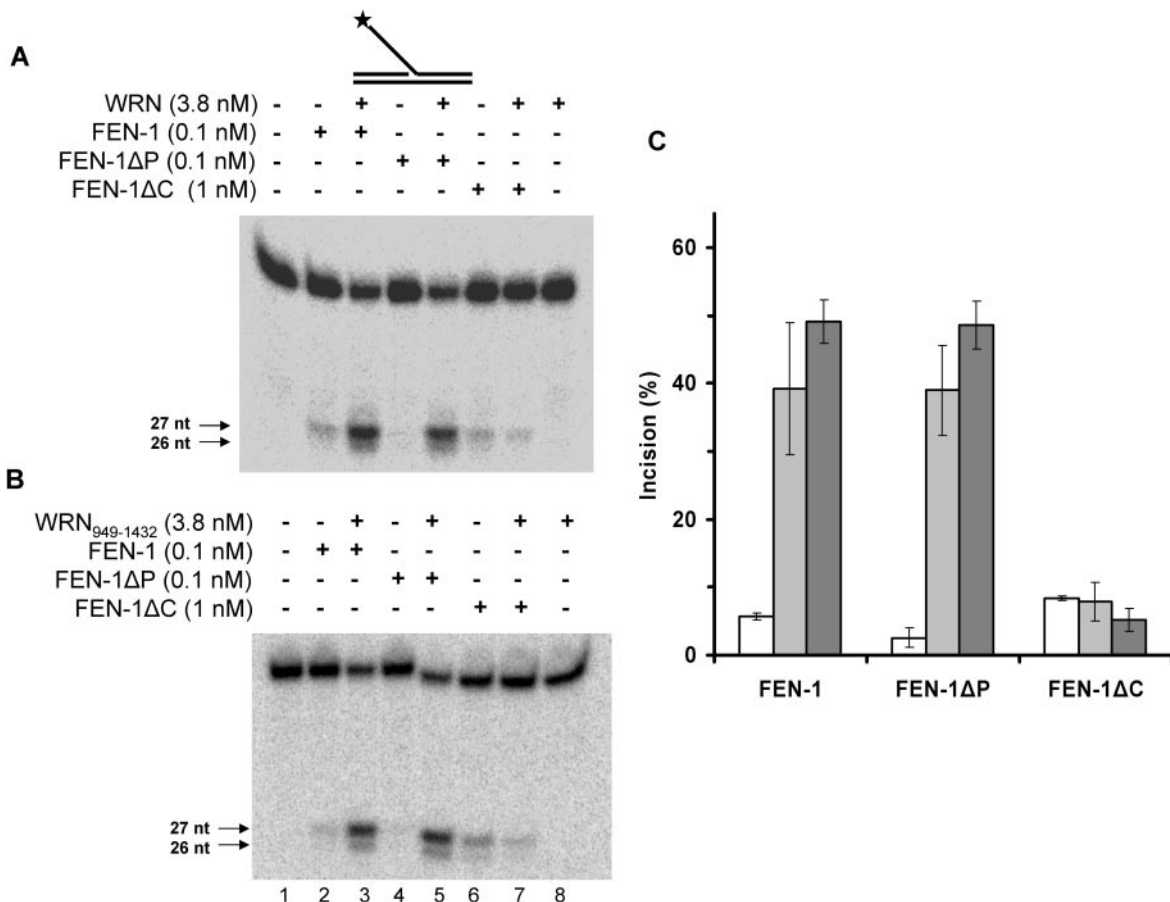


Figure 3. WRN-interacting region of FEN-1 is required for WRN stimulation of FEN-1 endonucleolytic cleavage of 5' flap substrate. Reaction mixtures (20 μ l) containing 10 fmol of the 26 nt 5' flap substrate, the specified concentrations of wild-type FEN-1, FEN-1ΔC or FEN-1ΔP, and either WRN (A) or WRN₉₄₉₋₁₄₃₂ (B) as indicated were incubated at 37°C for 15 min under standard conditions as described in Materials and Methods. Products were resolved on 20% polyacrylamide denaturing gels. Phosphorimages of typical gels are shown. For each gel: lane 1, no enzyme; lanes 2, 4 and 6 are wild-type FEN-1, FEN-1ΔP and FEN-1ΔC, respectively; lanes 3, 5 and 7 are wild-type FEN-1, FEN-1ΔP and FEN-1ΔC, respectively, in the presence of WRN (A) or WRN₉₄₉₋₁₄₃₂ (B); lane 8, WRN (A) or WRN₉₄₉₋₁₄₃₂ (B) alone. (C) Per cent incision (mean value of at least three independent experiments with SDs indicated by error bars). Quantitative data are shown for incision reactions with FEN-1, FEN-1ΔP or FEN-1ΔC alone (open bars), in the presence of WRN (light gray bars) or in the presence of WRN₉₄₉₋₁₄₃₂ (gray bars).

synergistic effect of both WRN and PCNA on FEN-1 incision might be detected. Since FEN-1 stimulation by PCNA is a diffusion-limited process that requires a large stoichiometric excess of PCNA (35,36), we used 3600 fmol PCNA which stimulated FEN-1 cleavage 3-fold (Figure 5A, lanes 2 and 4, and Figure 5B). In contrast, WRN stimulates FEN-1 cleavage significantly more effectively than PCNA (19); consequently, only 80 fmol WRN was used, resulting in 5.7-fold stimulation of FEN-1 cleavage (Figure 5A, lanes 2 and 3, and Figure 5B). The combined presence of WRN and PCNA with FEN-1 resulted in a maximal (12.7-fold) stimulation of FEN-1 cleavage (Figure 5A, lane 5, and Figure 5B). These results suggest that WRN and PCNA do not interfere with each other in their ability to stimulate FEN-1 cleavage.

The C-terminal tail of FEN-1 is required for the functional interaction with Bloom syndrome helicase

To address the importance of the physical interaction between BLM and FEN-1 for the functional interaction, we examined the ability of BLM to stimulate the cleavage activity of the FEN-1 deletion mutant lacking the BLM-interacting domain

(FEN-1ΔC). The results from FEN-1 incision assays demonstrate that the cleavage activity of FEN-1ΔC was not stimulated by BLM protein on a 26 nt 5' flap substrate (Figure 6A, lanes 6 and 7, and Figure 6B). In control reactions, BLM stimulated the cleavage activity of full-length FEN-1 by 16-fold (Figure 6A, lanes 2 and 3, and Figure 6B). We also tested the effect of BLM on the cleavage activity catalyzed by FEN-1ΔP, which lacks the PCNA interaction domain and found that its cleavage activity was stimulated similar to that observed for the wild-type FEN-1. These results indicate that the BLM-interacting region of FEN-1, but not the PCNA binding domain of FEN-1, is required for BLM to stimulate FEN-1 endonucleolytic cleavage of the 5' flap substrate.

WRN stimulates FEN-1 cleavage of its preferred double-flap substrate by interacting with the FEN-1 C-terminal tail and facilitating FEN-1 binding to the DNA structure

Recent evidence suggests that the physiological substrate of FEN-1 during DNA replication is a double-flap structure with equilibrating 3' and 5' single-stranded DNA tails that arises

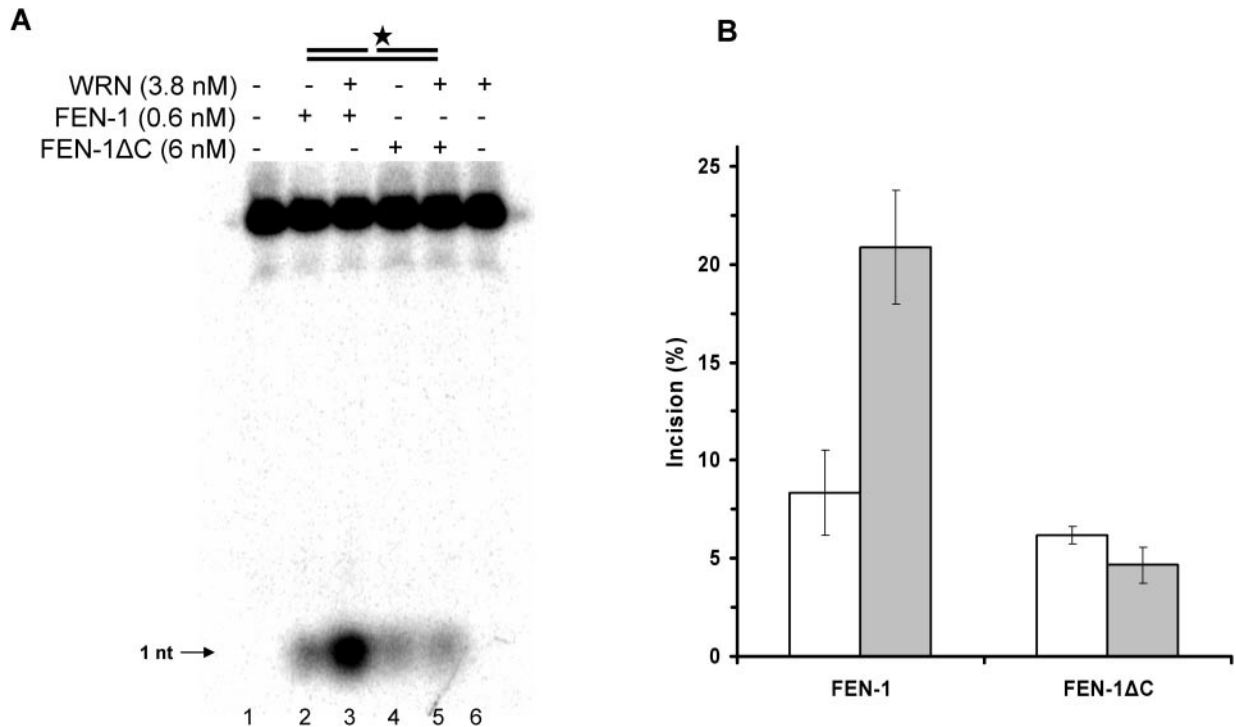


Figure 4. WRN-interacting region of FEN-1 is required for WRN stimulation of FEN-1 exonucleolytic cleavage of nicked duplex substrate. Reaction mixtures (20 μ l) containing 10 fmol of the nicked duplex DNA substrate, the specified concentrations of wild-type FEN-1 or FEN-1ΔC, and WRN as indicated were incubated at 37°C for 15 min under standard conditions as described in Materials and Methods. Products were resolved on 20% polyacrylamide denaturing gels. (A) Phosphorimage of a typical gel is shown. Lane 1, no enzyme; lanes 2 and 4 are wild-type FEN-1 and FEN-1ΔC, respectively; lanes 3 and 5 are wild-type FEN-1 and FEN-1ΔC, respectively, in the presence of WRN. (B) Per cent incision (mean value of at least three independent experiments with SD indicated by error bars). Quantitative data are shown for incision reactions with FEN-1 or FEN-1ΔC alone (open bars) or in the presence of WRN (light gray bars).

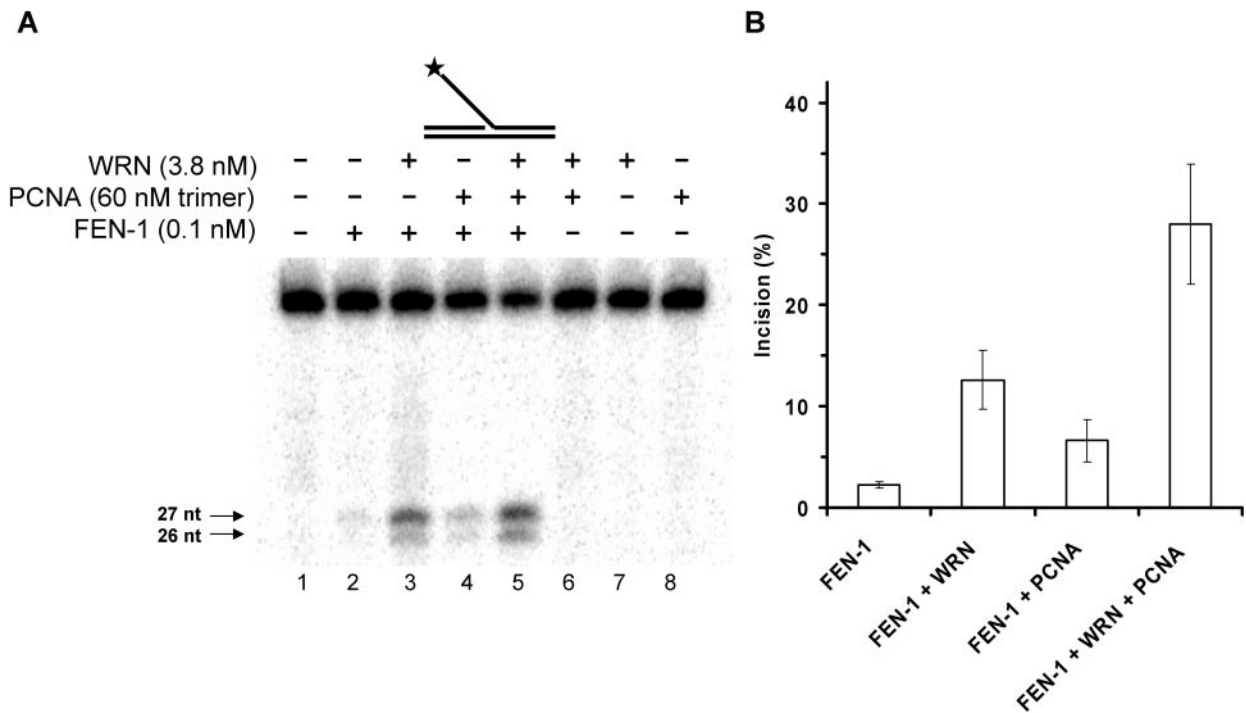


Figure 5. Combined effect of WRN and PCNA on stimulation of FEN-1 cleavage. Reaction mixtures (20 μ l) containing 10 fmol of the 26 nt 5' flap substrate and the indicated concentrations of FEN-1, WRN and/or PCNA were incubated at 37°C for 15 min under standard conditions as described in Materials and Methods. Products were resolved on 20% polyacrylamide denaturing gels. (A) Phosphorimage of a typical gel is shown. Lane 1, no enzyme; lane 2, FEN-1; lane 3, FEN-1 + WRN; lane 4, FEN-1 + PCNA; lane 5, FEN-1 + WRN + PCNA; lane 6, WRN + PCNA; lane 7, WRN; lane 8, PCNA. (B) Per cent incision (mean value of at least three independent experiments with SD indicated by error bars).

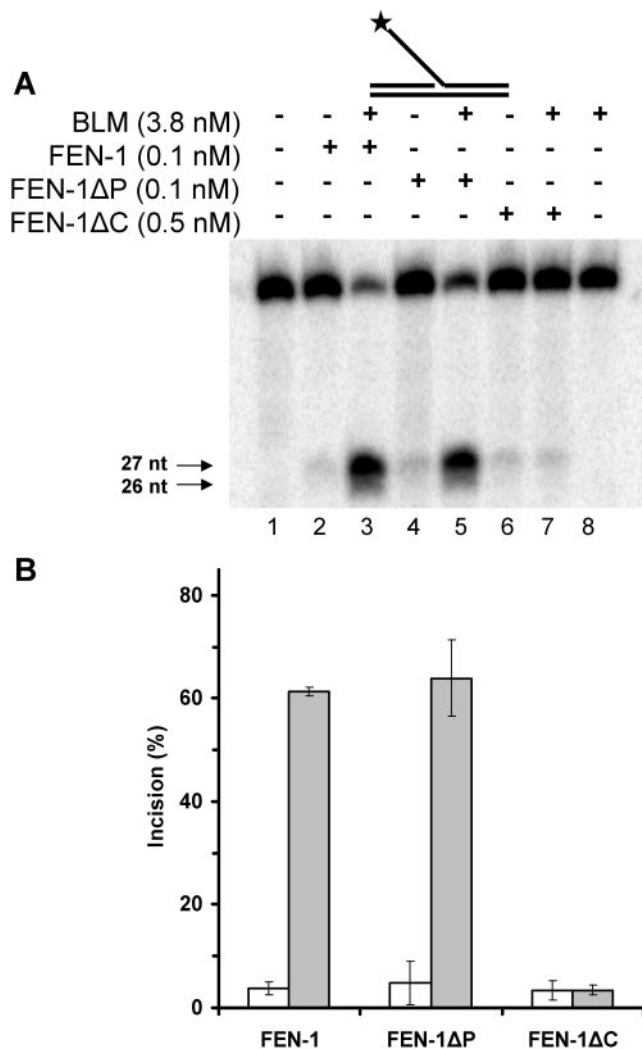


Figure 6. BLM-interacting region of FEN-1 is required for BLM stimulation of FEN-1 endonucleolytic cleavage of 5' flap substrate. Reaction mixtures (20 μ l) containing 10 fmol of the 26 nt 5' flap substrate, the specified concentrations of wild-type FEN-1, FEN-1 Δ C or FEN-1 Δ P, and BLM were incubated at 37°C for 15 min under standard conditions as described in Materials and Methods. Products were resolved on 20% polyacrylamide denaturing gels. (A) Phosphorimage of a typical gel is shown. Lane 1, no enzyme; lanes 2, 4 and 6 are wild-type FEN-1, FEN-1 Δ P and FEN-1 Δ C, respectively; lanes 3, 5 and 7 are wild-type FEN-1, FEN-1 Δ P and FEN-1 Δ C, respectively, in the presence of BLM; lane 8, BLM alone. (B) Per cent incision (mean value of at least three independent experiments with SD indicated by error bars). Quantitative data are shown for incision reactions with FEN-1 or FEN-1 Δ C alone (open bars) or in the presence of BLM (light gray bars).

after strand displacement DNA synthesis catalyzed by a DNA polymerase (37,38). FEN-1 preferentially cleaves the double-flap substrate with a 1 nt 3' tail at a precise position 1 nt into the downstream annealed region, allowing the 3' tail to anneal and generate a nick suitable for ligation. Because the double flap was proposed to be the cellular substrate of FEN-1, and *in vivo* evidence suggests that WRN has a role in DNA replication by its interaction with FEN-1, we investigated the mechanism for WRN stimulation of FEN-1 cleavage of the double-flap structure. The results in Figure 7 show that WRN stimulated wild-type FEN-1 cleavage of the double-flap substrate by 6.5-fold, whereas no stimulatory effect by WRN was observed

for the FEN-1 Δ C enzyme. Thus, as observed for the fixed 5' flap substrate, the ability of WRN to stimulate FEN-1 cleavage of the double-flap substrate was dependent on the presence of the C-terminal region of FEN-1 that interacts with WRN. We also observed that the WRN₉₄₉₋₁₄₃₂ fragment was unable to stimulate FEN-1 Δ C, but retained the ability to enhance cleavage of the double flap by wild-type FEN-1 (data not shown).

The previously published data indicated that the C-terminal tail of FEN-1 with which WRN or BLM helicases interact plays a role in DNA binding (28). These observations suggested to us that WRN may affect FEN-1 binding to its DNA substrate through its interaction with the C-terminal region of FEN-1. To experimentally address this hypothesis, we tested the effect of WRN on FEN-1 binding to the double-flap substrate using either the wild-type FEN-1 or FEN-1 Δ C. As shown by gel-shift analysis, a significantly greater amount of the double-flap substrate was bound at 120 nM FEN-1 compared with 60 nM FEN-1 (Figure 7C, lane 6 versus 2). The presence of WRN in the FEN-1 binding mixtures enhanced the amount of the shifted FEN-1:DNA species (Figure 7C, lanes 3–5). WRN alone did not shift the double-flap substrate (Figure 7C, lanes 7–9) to the position of the FEN-1:DNA complex. We next examined the effect of WRN on the ability of FEN-1 Δ C to bind the double flap. As shown in Figure 7D, WRN did not improve FEN-1 Δ C binding to the substrate at WRN protein concentrations in which wild-type FEN-1 binding to the double flap was effectively enhanced. These results indicate that WRN helps FEN-1 bind to its preferred double-flap substrate and that the C-terminal tail of FEN-1 that physically binds to WRN is necessary not only for WRN to stimulate FEN-1 cleavage of the double flap but also WRN enhancement of FEN-1 binding to the structure.

WRN retains the ability to stimulate acetylated FEN-1

It was reported recently that FEN-1 can be acetylated by p300 and that acetylation down-regulates FEN-1 incision activity (26). Three of the four acetylated lysine residues of FEN-1 were mapped to the extreme C-terminus which overlaps with the WRN binding site of FEN-1 (26). To assess the importance of FEN-1 acetylation status for the WRN–FEN-1 functional interaction, we examined the ability of WRN protein to stimulate acetylated FEN-1 *in vitro*. Under conditions in which FEN-1 is acetylated by p300 (Figure 8A), the FEN-1 cleavage reaction on the 26 nt 5' flap substrate is significantly reduced (Figure 8B), consistent with earlier observations that FEN-1 cleavage activity on a 20 nt 5' flap structure is reduced ~6-fold by FEN-1 acetylation (26). As shown in Figure 8B, WRN retains the ability to stimulate acetylated FEN-1. From quantitative analysis of the FEN-1 incision data it was determined that WRN (100 fmol) stimulated acetylated FEN-1 cleavage was from 5 to 43% at 40 fmol FEN-1 (Figure 8C). The 8.5-fold increase in cleavage activity by acetylated FEN-1 in the presence of WRN is very comparable with the fold stimulation of incision by unacetylated FEN-1 when WRN is present (Figure 3). To assess if FEN-1 acetylation modulates its ability to bind WRN, we performed ELISA assays with unacetylated or acetylated FEN-1 and WRN. The results, shown in Figure 8D, demonstrate that WRN retains its ability to physically interact with FEN-1 in its acetylated form similar

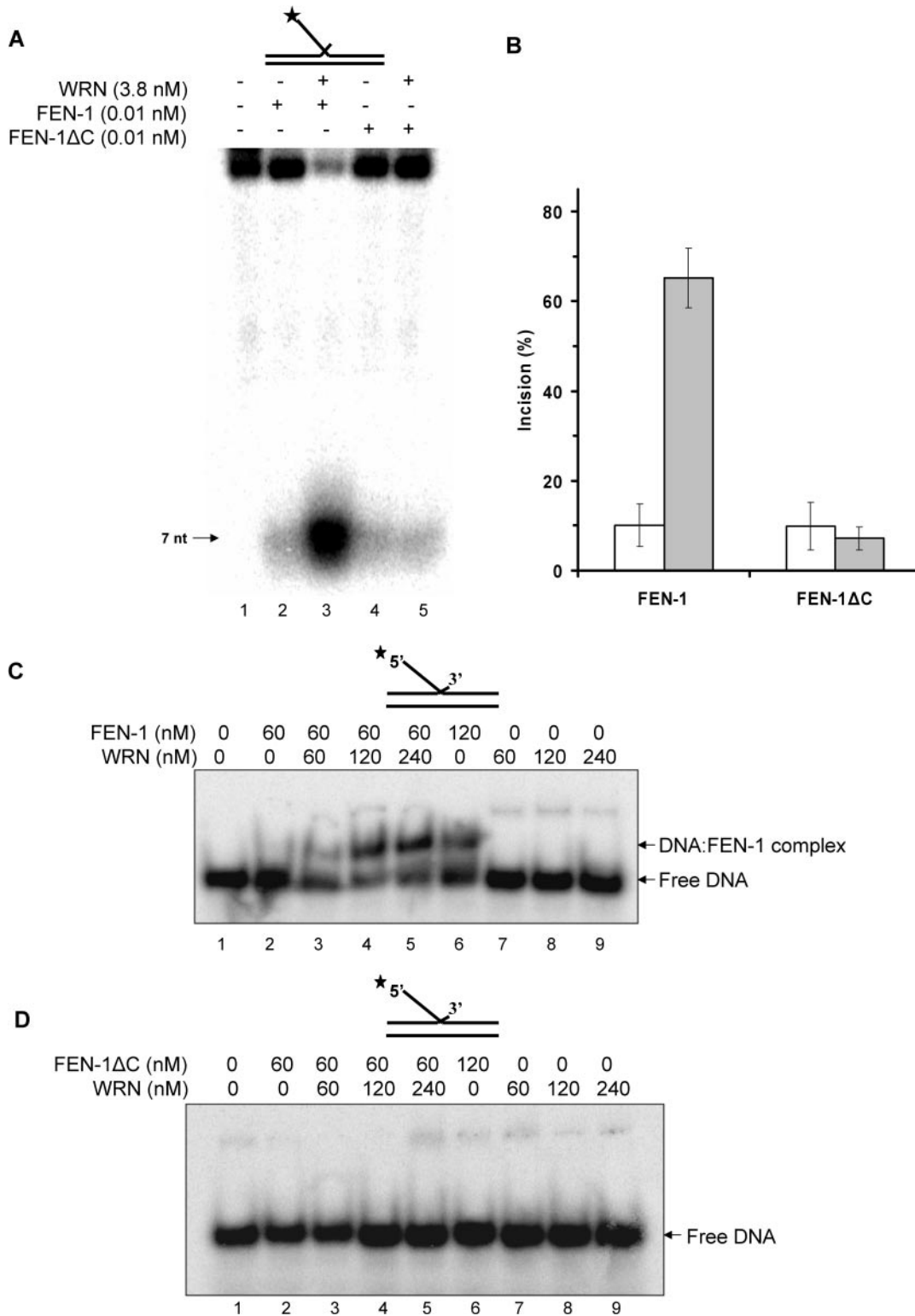


Figure 7. WRN facilitates FEN-1 binding to the double-flap DNA substrate through its interaction with the C-terminal tail of FEN-1. (A and B) Reaction mixtures (20 μ l) containing 10 fmol of the double flap DNA substrate, the specified concentrations of wild-type FEN-1 or FEN-1 Δ C, and WRN as indicated were incubated at 37°C for 15 min under standard conditions as described in Materials and Methods. Products were resolved on 20% polyacrylamide denaturing gels. (A) Phosphorimage of a typical gel is shown. Lane 1, no enzyme; lanes 2 and 4 are wild-type FEN-1 and FEN-1 Δ C, respectively; lanes 3 and 5 are wild-type FEN-1 and FEN-1 Δ C, respectively, in the presence of WRN. (B) Per cent incision (mean value of at least three independent experiments with SDs indicated by error bars). Quantitative data are shown for incision reactions with FEN-1 or FEN-1 Δ C alone (open bars) or in the presence of WRN (light gray bars). (C and D) Binding mixtures (20 μ l) containing 10 fmol of the double flap DNA substrate, the specified concentrations of wild-type FEN-1 (C) or FEN-1 Δ C (D), and WRN as indicated were incubated at 37°C for 15 min as described in Materials and Methods. Products were resolved on native 5% polyacrylamide gels. Phosphorimages of typical gels are shown.

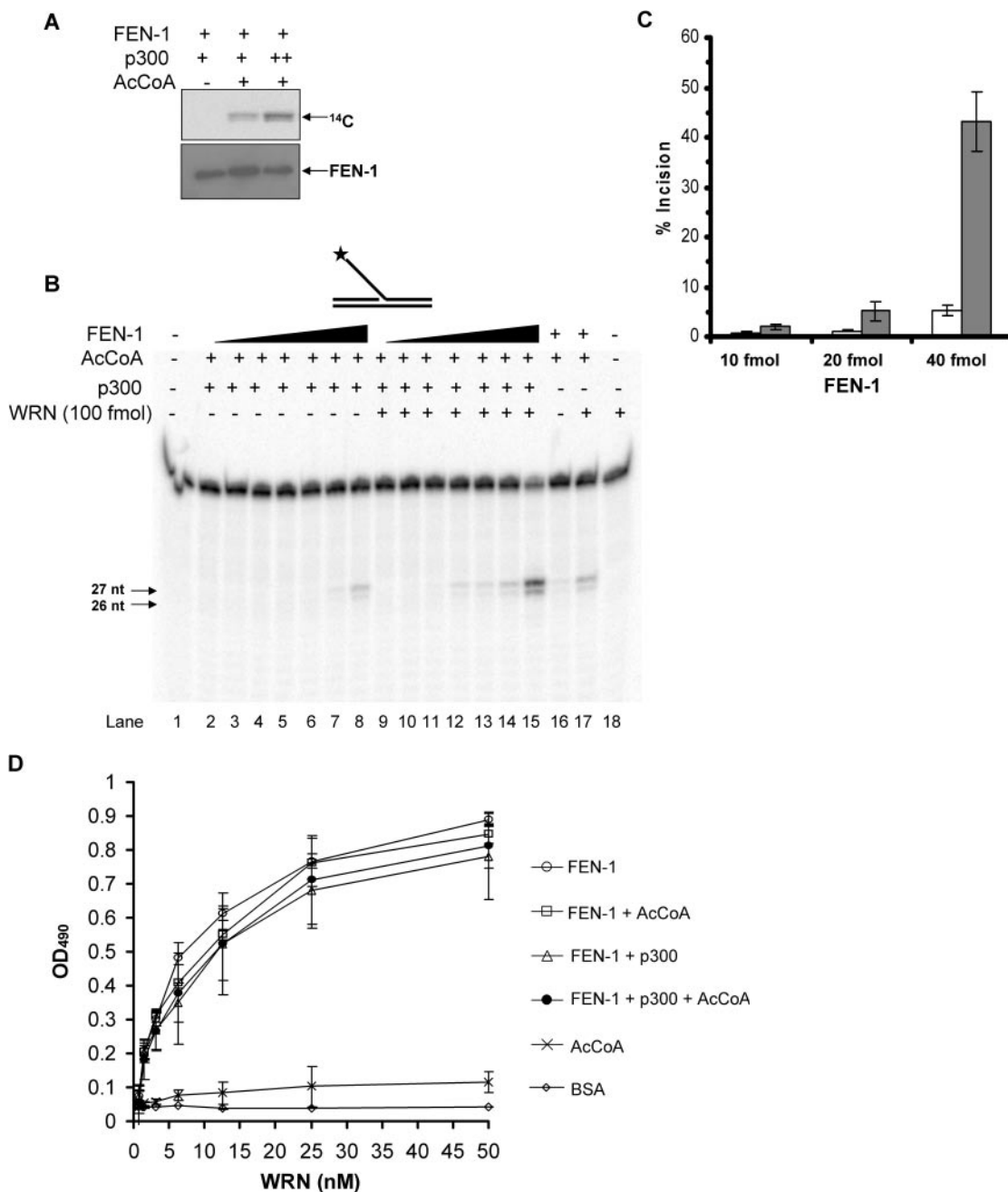


Figure 8. WRN retains the ability to physically bind and stimulate acetylated FEN-1. (A) Purified recombinant FEN-1 was acetylated *in vitro* by incubating with p300-HAT and [¹⁴C]acetyl coenzyme A (AcCoA) as described in Materials and Methods and resolved on SDS denaturing 10% polyacrylamide gel. The gel was exposed to X-ray film and developed by autoradiography (upper panel) or stained with Coomassie (lower panel). (B) Reaction mixtures (20 μ l) containing 10 fmol of the 26 nt 5' flap substrate, acetylated or unacetylated FEN-1 (0.625, 1.25, 2.5, 5, 10, 20 and 40 fmol) and WRN (100 fmol) were incubated at 37°C for 15 min under standard conditions as described in Materials and Methods. Products were resolved on 20% polyacrylamide denaturing gels. A phosphorimage of a typical gel is shown. Lanes 16 and 17 show the products of cleavage reactions containing 0.625 fmol FEN-1 (with AcCoA but lacking p300) in the absence and presence of WRN, respectively. (C) Per cent incision (mean value of at least three independent experiments with SD indicated by error bars from experiments as conducted in (B)). Quantitative data are shown for incision reactions with acetylated FEN-1 alone (open bars) or in the presence of WRN (light gray bars). (D) FEN-1 that had been preincubated with or without p300-HAT and/or AcCoA as indicated was coated onto ELISA plates. Following blocking with 3% BSA, the wells were incubated with increasing concentrations of purified recombinant WRN (0–50 nM) for 1 h at 30°C, and bound WRN was detected by ELISA using a rabbit polyclonal antibody against WRN followed by incubation with secondary HRP-labeled antibodies and OPD substrate. Data points are the mean of three independent experiments performed in duplicate with SDs indicated by error bars.

to that observed with unacetylated FEN-1. From the physical and functional assays, we can conclude that FEN-1 acetylation does not interfere with the ability of FEN-1 to interact with WRN.

The ability of WRN to physically and functionally interact with acetylated FEN-1 suggested that the lysine residues in the WRN-interacting region of FEN-1 that have been shown to be acetylated (26) may not be critical for the WRN interaction. To

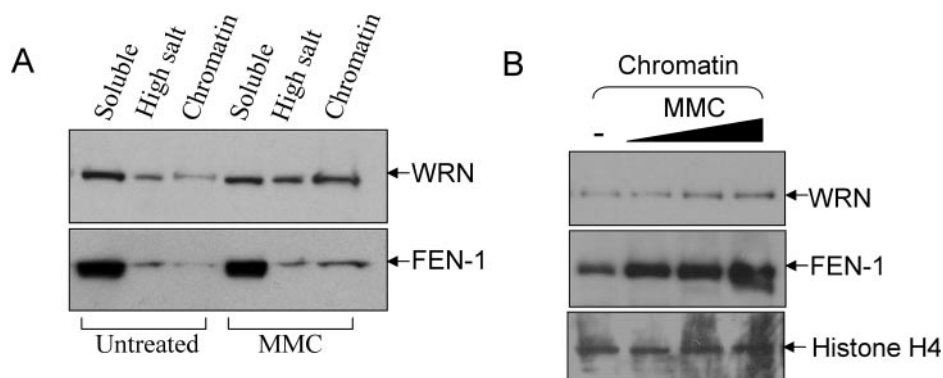


Figure 9. Enriched association of WRN–FEN-1 in the chromatin fraction after DNA damage. (A) Greater fraction of WRN and FEN-1 is found in the chromatin fraction after exposure of cells to MMC. Subnuclear fractionation was performed as described in Materials and Methods. Western blot detection of WRN and FEN-1 in different subnuclear fractions of HeLa cells treated with or without MMC (0.5 µg/ml). (B) Enrichment of WRN and FEN-1 in the chromatin fraction is dependent on MMC dose. Western blot detection of WRN and FEN-1 in the chromatin fractions prepared from HeLa cells treated with or without 0.25, 0.5 or 1 µg/ml MMC. Bottom panel shows Histone H4 detected by western blot.

explore this further, we performed WRN binding assays with FEN-1 mutant proteins in which one or more of the C-terminal lysine residues shown to be acetylated by p300 were replaced with alanines (39). The results from ELISA assays, shown in Supplementary Figure 1, demonstrate that WRN physically binds to each of the FEN-1 lysine mutants similarly, and in a comparable fashion to wild-type FEN-1. From a Hill plot analysis of the ELISA data, it was determined that the apparent dissociation constant (K_d) values for the WRN interaction with the various FEN-1 lysine mutants are all similar to that reported for the interaction of wild-type FEN-1 with WRN (23). Collectively, the results indicate that the lysine residues in the C-terminus of FEN-1 do not play a critical role in the WRN interaction.

Increased association of WRN and FEN-1 in the chromatin fraction after DNA damage

The replication defects and sensitivity of WS cells to DNA damaging agents that introduce replication fork blocking lesions suggest a specialized role of WRN at stalled replication forks. Consistent with this idea, WRN cellular localization studies have shown that WRN exits nucleoli in response to various DNA damaging agents and co-localizes with several proteins including FEN-1, RPA, PCNA and RAD52 at arrested replication foci [for review see (17,18)]. The earlier observations that FEN-1 is localized to repair foci after DNA damage (40) and becomes closely associated with WRN at arrested replication forks (24) suggested that the two proteins would be associated with each other in the same subnuclear fraction after treatment of cells with the interstrand cross-linking agent mitomycin C (MMC) that blocks replication fork progression. Detergent extracted lysates from untreated or MMC-treated cells were separated into soluble, high salt or chromatin fractions and analyzed for the presence of WRN or FEN-1 by western blotting. The results from a representative experiment in Figure 9A show that a greater amount of both WRN and FEN-1 are found in the high salt and chromatin fractions upon MMC treatment. The amount of WRN or FEN-1 found in the chromatin fraction was dependent on the concentration of MMC in the cellular treatment (Figure 9B). To control for loading, the same blot was probed with antibody against

Histone H4 (Figure 9B, lower panel). We next performed WRN–FEN-1 co-immunoprecipitation experiments using an antibody directed against FEN-1 and either the chromatin or soluble fractions. As we showed previously (19), WRN and FEN-1 were co-immunoprecipitated from the soluble fraction. As shown in Supplementary Figure 2, WRN and FEN-1 were associated with each other in the chromatin fraction and an increased amount of WRN was associated with FEN-1 in the chromatin fraction of MMC-treated cells compared with the untreated cells. Taken together, these results and WRN–FEN-1 FRET analyses (24) suggest that WRN and FEN-1 become associated with each other in the chromatin fraction at arrested replication foci.

DISCUSSION

Mutations in the WRN or BLM helicases are associated with a number of replication defects that include impaired fork progression, accumulation of abnormal replication intermediates and aberrant homologous recombination [for a review see (41)]. The results from biochemical and biological studies suggest that the cellular interaction between WRN and FEN-1 is important when DNA processing events during replication are compromised. By mapping the interaction site on FEN-1 for WRN or BLM, we have gained further understanding to how the involvement of FEN-1 with either WRN or BLM might be coordinated with its associations with other DNA replication/repair proteins including PCNA. The fact that the WRN/BLM interaction site is immediately adjacent to the PCNA binding site suggests that the C-terminal tail of FEN-1 is a major site for the regulation of its catalytic activity through its protein partner interactions. The shared FEN-1 binding site for WRN and BLM suggests that the two RecQ helicases are probable to modulate FEN-1 incision by a similar mechanism. The precise temporal and spatial aspects of how WRN, BLM and PCNA coordinate their interactions with FEN-1 during the dynamic processes of DNA replication or repair remain to be fully understood. Nevertheless, the mapping studies show that it is possible for FEN-1 to interact with PCNA and WRN (or BLM) either separately or simultaneously to perform its functions.

Our previous studies to investigate the kinetic mechanism for WRN stimulation of FEN-1 cleavage demonstrated that the mechanism whereby WRN stimulates FEN-1 cleavage (42) is distinct from that proposed for the functional interaction between PCNA and FEN-1 (43). Since PCNA was shown to not interact with the WRN binding site (residues 363–380) (30), and WRN was shown in this study to not interact with the PCNA binding site (residues 328–355), the mapping results clearly indicate distinct binding sites for WRN and PCNA on FEN-1. The distinction between WRN and PCNA for their FEN-1 interaction sites is probable to be important for their respective mechanisms of action with FEN-1 during DNA processing.

PCNA is required for the structural integrity of the replication fork and its normal progression during DNA synthesis of the replicating chromosome. One possibility is that WRN or BLM may be utilized as substitutes for PCNA in their interaction with FEN-1 at sites of DNA damage repair. Alternatively, PCNA may collaborate with WRN (or BLM) to stimulate FEN-1 cleavage, as suggested by the *in vitro* studies in this work. The disruption of the FEN-1–PCNA interaction by competitive binding of p21 to the FEN-1 interaction site of PCNA (44) may serve to enable WRN or BLM to interact with FEN-1 when normal replication fork progression is perturbed.

In contrast to PCNA, WRN and BLM share the same FEN-1 interaction site. This discovery coupled with the fact that WRN and BLM interact with FEN-1 through a conserved non-catalytic region that resides C-terminal to the RecQ helicase core domain suggests a conserved protein interaction interface of WRN and BLM with FEN-1. The crystal structure of the conserved catalytic core of the *E.coli* RecQ helicase revealed that it comprises four independently folded structural subdomains: two of them form the helicase region and the two RQC subdomains constitute a Zn²⁺ binding domain and winged helix (45). Interestingly, the winged helix sub-domain aligns with the first 110 residues of the FEN-1 interaction domain of WRN (WRN_{949–1092}) as well as a segment of the BLM domain fragment (residues 1076–1217) that mediates the FEN-1 interaction. This suggests that the FEN-1 interaction domain of WRN or BLM protein folds independently from other regions of the protein and may be capable of functioning independently. Consistent with this notion, WRN or BLM fragments harboring the FEN-1 interaction domain are able to bind the minimal FEN-1 sequence, residues 363–380, that mediates the interaction of FEN-1 with full-length WRN or BLM proteins.

What factors might be important in regulating the interaction of FEN-1 with its protein partners and how might this be important *in vivo*? In human cells, the post-translational modification state of FEN-1 or its interacting partner may regulate its role in replication or cell cycle progression. Acetylation of FEN-1 by the transcriptional coactivator p300 is enhanced upon UV treatment of human cells, and significantly reduces FEN-1's DNA binding and nuclease activity (26). Three of the four identified acetylated lysine residues were located in the extreme C-terminal tail of FEN-1 that is the binding site for WRN. Despite the fact that FEN-1 is acetylated in the WRN binding site, the acetylated form of FEN-1 can be optimally stimulated by WRN.

It was proposed that the unique C-terminal region of FEN-1 might be important to regulate its enzymatic activity through modulation of DNA substrate binding. This hypothesis is

supported by the demonstration that WRN enhances FEN-1 binding to its preferred double-flap DNA substrate. The C-terminal region of FEN-1 is not present in prokaryotic organisms (Archaea), suggesting a specialized function in DNA metabolism. The unique C-terminal tail of FEN-1 is an important site for binding of WRN or BLM helicases, and the absence of the tailored interaction may contribute to the genomic instability of WS and Bloom syndrome.

The results from the WRN/BLM-FEN-1 mapping studies may have some bearing on understanding the impact of genetic mutations on human disease. Single nucleotide polymorphisms in FEN-1 have been identified in the human population (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=2237&chooseRs=all), including a Phe/Leu variation at residue 376 in the C-terminal tail of FEN-1 where BLM or WRN proteins interact with FEN-1 (refSNP number rs7931165). Biochemical and genetic characterization of the 376 variants and other FEN-1 polymorphic variants will be informative as their importance in human disease is assessed. In addition, two of the known BLM clinical missense mutations (46,47) reside in the RecQ-Ct region that is responsible for the FEN-1 physical and functional interaction (25). Future work will address the importance of genetic variations that impact the interactions of RecQ helicases with Rad2 nucleases.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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