Werner syndrome protein interacts with human flap endonuclease 1 and stimulates its cleavage activity

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Werner syndrome (WS) is a human premature aging disorder characterized by chromosomal instability. The cellular defects of WS presumably reflect compromised or aberrant function of a DNA metabolic pathway that under normal circumstances confers stability to the genome. We report a novel interaction of the WRN gene product with the human 5’ flap endonuclease 5′–3′ exonuclease (FEN-1), a DNA structure-specific nuclease implicated in DNA replication, recombination and repair. WS protein (WRN) dramatically stimulates the rate of FEN-1 cleavage of a 5’ flap DNA substrate. The WRN–FEN-1 functional interaction is independent of WRN catalytic function and mediated by a 144 amino acid domain of WRN that shares homology with RecQ DNA helicases. A physical interaction between WRN and FEN-1 is demonstrated by their co-immunoprecipitation from HeLa cell lysates and affinity pull-down experiments using a recombinant C-terminal fragment of WRN. The underlying defect of WS is discussed in light of the evidence for the interaction between WRN and FEN-1.

Keywords: flap endonuclease 1/genomic instability/helicase/replication/Werner syndrome

Introduction

Werner syndrome (WS) is an autosomal recessive disorder that displays symptoms of premature aging after adolescence (Martin, 1978). WS cells exhibit replication defects (Martin et al., 1970; Takeuchi et al., 1982; Hanaoka et al., 1985; Salk et al., 1985; Poot et al., 1992), genomic instability (Salk et al., 1981, 1985; Fukuchi et al., 1989) and altered telomere dynamics (Hisama et al., 2000; Ouellette et al., 2000; Wyllie et al., 2000). An anti-recombinogenic role of WRN has been suggested (Yamagata et al., 1998; Constantinou et al., 2000); however, the basis for the genomic instability in WS is not understood.

The gene defective in WS, designated WRN, encodes a nuclear (Matsumoto et al., 1997) 1432 amino acid protein with the seven conserved motifs found in the RecQ family of DNA helicases (Yu et al., 1996; for review see Mohaghegh and Hickson, 2001) (Figure 1). WRN is a DNA-dependent ATPase and 3’ to 5’ helicase (Gray et al., 1997; Suzuki et al., 1997). The protein sequence of WRN also contains a region of conserved exonuclease motifs (Moser et al., 1997) (Figure 1), and possesses a 3′ to 5′ exonuclease activity (Huang et al., 1998; Kamath-Loeb et al., 1998; Shen et al., 1998). The catalytic activities of WRN suggest that a pathway of DNA metabolism is defective in WS. A role of WRN in DNA replication and/or repair is suggested by a number of its protein interactions (Shen et al., 1998; Bladerer et al., 1999; Brosh et al., 1999, 2001; Lebel et al., 1999; Spillare et al., 1999; Cooper et al., 2000; Kamath-Loeb et al., 2000; Kawabe et al., 2000; Li and Comai, 2000; Szekely et al., 2000) (Figure 1A). Precisely how these protein interactions are important in a biological setting remains to be established.

The evidence implicating 5′ flap endonuclease 5′–3′ exonuclease (FEN-1) in DNA replication, repair and the maintenance of genomic stability suggested that WRN may interact with FEN-1 to facilitate its function. FEN-1 is required during Okazaki fragment processing (Bambara et al., 1997) and long patch base excision repair (BER) (Klungland and Lindahl, 1997; Kim et al., 1998). FEN-1 is a DNA structure-specific nuclease that cleaves 5′ flap single-stranded (ss)DNA at the single strand–double strand junction (reviewed in Lieber, 1997). FEN-1 is active as a 5′ to 3′ exonuclease at nicks in duplex DNA and also catalytically removes the 5′ terminal RNA mononucleotides, the latter process thought to be important in Okazaki fragment processing (Waga and Stillman, 1998).

The FEN-1 homolog RAD27 in Saccharomyces cerevisiae plays an important role in the maintenance of genome stability (Johnson et al., 1995; Sommers et al., 1995; Vallen and Cross, 1995; Tishkoff et al., 1997; Freudenreich et al., 1998; Kokoska et al., 1998; Schweitzer and Livingston, 1998; Gary et al., 1999a), telomere stability (Parenteau and Wellinger, 1999), response to DNA damage (Reagan et al., 1995; Sommers et al., 1995) and non-homologous end-joining (NHEJ) (Wu et al., 1999). Thus genomic instability persists in vivo when FEN-1 is either absent or its cleavage activity is blocked by DNA secondary structure.

Proliferating cell nuclear antigen (PCNA) binds FEN-1 (Li et al., 1995; Wu et al., 1996) and stimulates FEN-1 nuclease activity (Tom et al., 2000). Elimination of PCNA binding by a site-specific mutation in RAD27 did not significantly increase genetic instability, recombination or methyl methanethiosulfonate sensitivity (Gary et al., 1999b), suggesting that redundant protein interactions/enzyme activities may be important in vivo. Dna2, a DNA helicase and endonuclease that physically and genetically interacts
with RAD27, may play a direct role in Okazaki fragment maturation in conjunction with FEN-1 (Bae and Seo, 2000). However, no functional homologs for Dna2 have been identified in mammalian systems.

We report that WRN interacts physically with FEN-1 and stimulates FEN-1 cleavage. The functional interaction is independent of WRN catalytic activities and mediated by a C-terminal region of WRN. WRN and FEN-1 are
likely to act together during DNA replication and/or repair. Defects in the WRN–FEN-1 interaction may contribute to the genomic instability of WS.

Results

Physical interaction between WRN and FEN-1

The C-terminal domain of WRN does not have any known catalytic activities, but binds to some of the WRN-interacting proteins (Figure 1). We established a pull-down assay to determine a possible association of glutathione S-transferase (GST)–WRN<sub>949-1432</sub> (Figure 1) with FEN-1 from human nuclear extract (NE). FEN-1 was coprecipitated with GST–WRN<sub>949-1432</sub> (Figure 2A, lane 3) by comparison with the input (lane 1). In the absence of NE input, a specific band migrating at the position of FEN-1 was not detected (Figure 2A, lane 5). Both GST and GST–WRN<sub>1072-1236</sub> were unable to coprecipitate FEN-1 (Figure 2A, lanes 2 and 4). There was some cross-reactivity of the anti-FEN-1 antibody to bacterial proteins migrating at higher molecular weight (Figure 2A, lanes 3–6). These results demonstrate that FEN-1 can be specifically precipitated by the GST–WRN<sub>949-1432</sub> fragment. To address the possibility that the interaction between GST–WRN<sub>949-1432</sub> and FEN-1 may have been indirectly mediated by other NE proteins, purified recombinant FEN-1 was incubated with the GST–WRN<sub>949-1432</sub> affinity resin. The results demonstrate that FEN-1 is precipitated by the GST–WRN<sub>949-1432</sub> affinity beads (Figure 2B, lane 2), but not by GST resin (Figure 2B, lane 1). The FEN-1–GST–WRN<sub>949-1432</sub> interaction was resistant to DNase I (10 μg/ml), indicating that binding was not DNA mediated (data not shown).

To determine whether full-length WRN interacts with FEN-1, we tested for full-length recombinant WRN to bind FEN-1–Sepharose beads. As shown in Figure 2C, WRN remained bound through successive washes (lane 2) and was eluted from the FEN-1–Sepharose beads (lane 4). Twenty percent of the purified WRN input was bound by the FEN-1–Sepharose beads. WRN failed to effectively bind to the bovine serum albumin (BSA)–Sepharose beads as detected in the eluted fraction (Figure 2C, lane 3). These studies indicate that full-length WRN binds directly to FEN-1.

To determine whether endogenous WRN and FEN-1 interact in vivo, co-immunoprecipitation experiments from HeLa whole-cell extracts were performed using a polyclonal antibody against the WRN N-terminus (Figure 2D). FEN-1 was effectively precipitated by the WRN antibody (Figure 2D, lane 3) and failed to be detected when the WRN antibody was omitted from the immunoprecipitation (lane 1). As a negative control, similar experiments were performed using WS cells (AG11395). FEN-1 failed to be immunoprecipitated by the WRN antibody (Figure 2D, lane 5) despite its presence in the AG11395 cell lysate (lane 4). These results demonstrate that WRN and FEN-1 can be co-immunoprecipitated from HeLa NE.

WRN stimulates the FEN-1 cleavage reaction

The physical interaction between WRN and FEN-1 suggested that the two proteins might modulate the catalytic activity of each other. No significant effect of FEN-1 on WRN ATPase or helicase activity was detected

Fig. 2. WRN and FEN-1 interact physically. (A) Beads with GST (lane 2), GST–WRN<sub>949-1432</sub> (lane 3) or GST–WRN<sub>1072-1236</sub> (lane 4) were incubated with 750 μg of HeLa NE. In control experiments, NE was omitted during binding with GST–WRN<sub>949-1432</sub> (lane 5) or GST–WRN<sub>1072-1236</sub> (lane 6). Western blotting was performed with anti-FEN-1 antibodies. The NE input (lane 1) corresponds to 10 μg. (B) Beads with GST (lane 1) or GST–WRN<sub>949-1432</sub> (lane 2) were incubated with 1.2 μg of recombinant FEN-1 purified from E.coli. In control experiments, FEN-1 was omitted during binding with GST–WRN<sub>949-1432</sub> (lane 3). Western blotting was performed with anti-FEN-1 antibodies. The FEN-1 input (lane 4) corresponds to 20 ng. (C) FEN-1–Sepharose specifically binds recombinant WRN. Purified WRN and FEN-1 were judged to be pure by DNA analysis using SYBR Green Stain (FMC Products). Western blot of the third wash (lanes 1 and 2) or eluted fractions (lanes 3 and 4) from binding reaction of WRN to FEN-1–Sepharose (lanes 2 and 4) or BSA–Sepharose (lanes 1 and 3) is shown. (D) WRN and FEN-1 coprecipitate from HeLa cell lysate using anti-WRN antibody as demonstrated by western blotting. Top panel, blot was probed with anti-FEN-1 antibody. Bottom panel, blot was probed with anti-WRN antibody. Lane 1, control precipitate from HeLa cell lysate in which WRN antibody was omitted from immunoprecipitation; lane 2, HeLa cell lysate input; lane 3, immunoprecipitate from HeLa cell lysate using WRN antibody; lane 4, AG11395 (WS−/−) cell lysate input; lane 5, immunoprecipitate from AG11395 cell lysate using WRN antibody; lane 6, purified His-tagged FEN-1, which migrates slightly higher than NE FEN-1.
(data not shown). To characterize the effect of WRN on FEN-1 cleavage, we utilized a 19 bp DNA substrate with a single unannealed 5' nucleotide adjacent to an upstream 25 bp duplex (1 nt 5' flap). The 1 nt 5' flap substrate was susceptible to FEN-1 cleavage that generated 1 and 2 nt products (Figure 3A, lane 2), as previously published (Tom et al., 2000). In the presence of 100 fmol of purified FEN-1, 5% of the substrate was incised (Figure 3A, lane 2, and B). In the presence of WRN (75 fmol), FEN-1 incised 55% of the flap substrate molecules (Figure 3A, lane 3, and B). Thus, at nearly equimolar concentrations of WRN and FEN-1, FEN-1 cleavage is stimulated 11-fold. Importantly, WRN alone did not catalyze significant cleavage of the 1 nt flap DNA substrate (Figure 3A, lane 4).

We subsequently analyzed FEN-1 cleavage as a function of WRN concentration under standard conditions (40 mM KCl) or in the absence of KCl, a more optimal condition for FEN-1 incision (Harrington and Lieber, 1994). A limiting amount of FEN-1 (10 fmol) was used such that cleavage of the 1 nt 5' flap is very low (~1%, 40 mM KCl, ~3%, no KCl) (Figure 3C, lane 3, and D). FEN-1 cleavage was reproducibly stimulated ~2-fold at a WRN amount of 20 fmol (Figure 3C, lane 6, and D) in the presence or absence of KCl. In the presence of 40 fmol of WRN, FEN-1 cleavage increased to 17 and 31% incision in the presence and absence of KCl, respectively (Figure 3C, lane 5, and D). At 80 fmol of WRN, product formation began to plateau at 20% for the FEN-1 cleavage reaction conducted in the presence of 40 mM KCl, whereas cleavage continued to increase to nearly 60% in the absence of KCl (Figure 3C, lane 4, and D).

Kinetic analysis of the FEN-1-catalyzed cleavage reaction on the 1 nt 5' flap substrate demonstrated a dramatic stimulation of the rate of FEN-1 incision in the presence of WRN (Figure 4A). In these experiments, an amount of WRN was used that we previously determined to achieve maximum stimulation of FEN-1 cleavage (Figure 3D). The level of FEN-1 used resulted in a low, but reproducibly detectable incision of 2% of the 10 fmol of DNA substrate in a 15 min reaction incubated at 37°C (in the absence of WRN) (Figure 4A). Stimulation of FEN-1 incision by WRN was detected at time points as short as 0.5–1 min (Figure 4B). Up to 3 min, FEN-1 cleavage in the absence of WRN was below 1%; however, in the presence of WRN, FEN-1 cleaved 12% of the DNA substrate (Figure 4B). FEN-1 cleavage in the presence and absence of WRN was linear with respect to time from 0.5–3 min ($R^2 = 1.0$ and 0.98, respectively). Linear regression analyses yielded reaction rates of 4.5 and 0.06 fmol product/min for the WRN + FEN-1 and FEN-1 only reactions, respectively. This represents an 82-fold rate increase when WRN is present. At 12 and 15 min, the FEN-1 cleavage reaction conducted in the presence of WRN achieved a plateau of 30–35% substrate incised.

**Fig. 3.** WRN stimulates FEN-1 cleavage activity. (A) Reactions (20 μl) containing 10 fmol of a 1 nt 5' flap DNA substrate, 100 fmol of FEN-1 and 75 fmol of WRN were incubated at 37°C for 15 min under standard conditions as described in Materials and methods. A phosphorimaging of a typical gel is shown. Substrate and cleavage products are as indicated. Lane 1, no enzyme; lane 2, FEN-1; lane 3, FEN-1 + wild-type WRN; lane 4, wild-type WRN. (B) % incision from (A) (mean value of three experiments) with standard deviation (SD) indicated by error bars. (C) Reactions (20 μl) containing 10 fmol of a 1 nt 5' flap DNA substrate, 10 fmol of FEN-1 and the indicated amounts of WRN were incubated in the absence or presence of 40 mM KCl as indicated at 37°C for 15 min. A phosphorimaging of a typical gel is shown. Lane 1, no enzyme; lane 2, 80 fmol of WRN; lane 3, FEN-1; lane 4, 80 fmol of WRN + FEN-1; lane 5, 40 fmol of WRN + FEN-1; lane 6, 20 fmol of WRN + FEN-1; lane 7, 10 fmol of WRN + FEN-1; lane 8, 5 fmol of WRN + FEN-1. (D) % incision from (C) (mean value of three experiments) with SD. Filled circles, no KCl; open circles, 40 mM KCl.
In contrast, FEN-1 alone only cleaved 1.5% of the substrate by the end of 15 min (Figure 4B).

WRN stimulates FEN-1 cleavage more effectively than either PCNA or replication protein A

Since replication protein A (RPA) and PCNA interact physically with WRN (Brosh et al., 1999; Lebel et al., 1999) and are capable of stimulating the FEN-1 cleavage reaction (Biswas et al., 1997; Tom et al., 2000), we tested WRN for the presence of either RPA or PCNA by western blot analysis. We examined the purity of recombinant WRN and it did not contain RPA or PCNA from insect cells (data not shown).

We then tested purified PCNA and RPA for stimulation of FEN-1 cleavage on the 1 nt 5’ flap substrate. At molar amounts of PCNA homotrimer or RPA heterotrimer equal to WRN monomer (80 fmol) that displayed a 10-fold stimulation of FEN-1 cleavage, we did not detect any significant stimulation of FEN-1 cleavage by either PCNA or RPA (Figure 5). Stimulation of FEN-1 incision was detected at much higher amounts of PCNA (900 fmol homotrimer) (data not shown). These findings are consistent with a previous report (Tom et al., 2000) that PCNA can stimulate FEN-1 cleavage of the same 1 nt 5’ flap substrate used in this study. However, a large amount of PCNA (25 000 fmol) was used in that study because FEN-1 stimulation by PCNA is a diffusion-limited process that requires a large stoichiometric excess of PCNA (Burgers and Yoder, 1993; Wu et al., 1996). On a per mole basis, WRN is a significantly more effective stimulator of FEN-1 cleavage on the 1 nt 5’ flap substrate than either human PCNA or RPA.

WRN may serve to shield the negative charge on the DNA, thereby permitting FEN-1 to bind, and thus cleave the substrate more easily. If so, then another DNA-binding protein such as Ku might also stimulate FEN-1 cleavage. We tested the effect of Ku86/70 heterodimer on FEN-1 cleavage and did not detect any stimulation at a range of Ku concentrations (10–500 fmol) under the same conditions that WRN effectively stimulated FEN-1 cleavage (data not shown).

Catalytic activities of WRN are not required for stimulation of FEN-1 incision

The ATPase, helicase or exonuclease activities of WRN may play a role in the functional interaction with FEN-1. To address this, we tested the effects of full-length recombinant WRN proteins (Figure 1) with site-directed mutations in the active sites of its catalytic domains on FEN-1 cleavage. The WRN-K577M mutant protein, devoid of ATPase or helicase activity (Gray et al., 1997; Brosh et al., 1999), was capable of stimulating the FEN-1 cleavage reaction similarly to wild-type WRN (Figure 6A, lanes 3 and 4). In control reactions, WRN-K577M alone did not yield the products (Figure 6A, lane 6). Thus, ATP hydrolysis/DNA unwinding are dispensable for the WRN–FEN-1 functional interaction, consistent with our finding that WRN stimulates FEN-1 cleavage of the 1 nt flap substrate in the absence or presence of ATP (data not shown).
To address the importance of WRN exonuclease activity in the WRN–FEN-1 functional interaction, we tested the ability of a WRN exonuclease defective mutant WRN-E84A (Huang et al., 1998; Cooper et al., 2000) to stimulate FEN-1 cleavage. As shown in Figure 6B, WRN-E84A retained the ability to stimulate FEN-1 incision (lane 4). In control reactions, WRN-E84A alone did not yield the cleavage products (Figure 6B, lane 6). These results indicate that the exonuclease activity of WRN is not required for the functional interaction between WRN and FEN-1.

**Mapping of the WRN domain that is important for stimulation of FEN-1 cleavage**

The ability of the catalytically defective mutant WRN proteins to stimulate FEN-1 cleavage suggested that the functional interaction is mediated by a direct protein interaction. The physical interaction between FEN-1 and the WRN C-terminus (Figure 2) raised the possibility that this region may stimulate FEN-1 cleavage by itself. The results shown in Figure 7 provide evidence that indeed this is the case. Using a limiting amount of FEN-1 (5 fmol), the cleavage reaction was stimulated 8.5-fold by GST–WRN949–1432 (Figure 7A, lanes 3 and 4, and B). Importantly, the GST–WRN1072–1236 fragment that did not bind to FEN-1 failed to stimulate FEN-1 incision (Figure 7A, lane 5). Using a 2-fold higher level of FEN-1 (10 fmol), 6% of the DNA substrate was cleaved (Figure 7A, lane 6, and B). At this FEN-1 level, GST–WRN949–1432 stimulated FEN-1 cleavage to 46% of the substrate incised (Figure 7A, lane 7, and B). GST–WRN1072–1236 again failed to stimulate FEN-1 cleavage (Figure 7A, lane 6), indicating that the functional interaction is specific to WRN residues 949–1432. Similar results were obtained using 20 fmol of FEN-1 (lanes 9–11), although the level of stimulation was not as great because a plateau of incision activity (~58%) was approached in the reactions containing FEN-1 and GST–WRN949–1432 (Figure 7B). A highly purified WRN fragment without the GST moiety (His-WRN949–1432) (Cooper et al., 2000) (Figure 1B) is also competent to physically and functionally interact with FEN-1 (see Supplementary figure 1, available at The EMBO Journal Online), suggesting that the interaction is not associated with GST and is specific to the WRN sequence 940–1432.
To further map the domain of WRN that mediates the functional interaction with FEN-1, several additional recombinant GST–WRN fragments were tested. As shown in Figure 8A, lane 4, GST–WRN949–1236, a shortened version of GST–WRN949–1432 that lacks 196 amino acids at the extreme C-terminus (Figure 1), stimulated FEN-1 incision of the 1 nt 5’ flap substrate 5-fold compared with the reaction containing FEN-1 only (Figure 8A, lane 2). In control reactions, GST–WRN949–1236 alone did not produce the FEN-1 incision products (Figure 8A, lane 9). The level of FEN-1 stimulation by GST–WRN949–1236 was comparable to that of GST–WRN949–1432 (Figures 7A, lane 7, and 8B), suggesting that the last 196 amino acids of GST–WRN949–1432 are dispensable for stimulation of FEN-1 cleavage. GST–WRN1072–1236 (Figures 8A, lane 5, and B, and 7) or GST (data not shown) failed to stimulate FEN-1 cleavage, attesting to the specificity of GST–WRN949–1236 in the functional interaction with FEN-1.

Since GST–WRN949–1236 was able to stimulate FEN-1 incision whereas GST–WRN1072–1236 failed, the domain of WRN necessary for functional interaction with FEN-1 might reside within residues 949–1072. To address this, we tested a GST–WRN recombinant fragment spanning residues 949–1092 (GST–WRN949–1092) (Figure 1). GST–WRN949–1092 was able to stimulate FEN-1 cleavage quite effectively (Figure 8A, lane 6). GST–WRN949–1092 alone did not produce the FEN-1 cleavage products (Figure 8A, lane 11). Compared with the reaction containing only FEN-1 (Figure 8A, lane 2) or FEN-1 + GST–WRN239–499 (lane 7), a control WRN fragment residing in the N-terminus (Figure 1), the level of FEN-1 cleavage in the presence of GST–WRN949–1092 was 6-fold greater (Figure 8B). The ability of the highly purified GST–WRN949–1236 and GST–WRN894–1092 fragments (Figure 1B) to stimulate FEN-1 cleavage indicates that the protein domain responsible for stimulating FEN-1 cleavage resides within the C-terminus of WRN. More specifically, a 144 amino acid domain of WRN (residues 949–1092) mediates the functional interaction with FEN-1.

**WRN stimulates FEN-1 cleavage of duplex DNA substrates containing a longer 5’ flap or nick**

The ability of WRN to stimulate FEN-1 cleavage of the 1 nt 5’ flap substrate raised the question of whether WRN can also affect the activity of FEN-1 on DNA substrates with longer 5’ flaps. These structures may be important reaction intermediates on the newly synthesized lagging strand during Okazaki fragment processing or during long patch BER. We tested WRN in the FEN-1 cleavage reaction of a 5 nt 5’ flap structure. In the presence of 10 fmol of FEN-1, 8% of the substrate was incised (Figure 9A, lane 2, and C). In the presence of WRN (75 fmol), FEN-1 incised 68% of the 5’ flap substrate molecules (Figure 9A, lane 3, and C), an 8.5-fold stimulation. Importantly, WRN alone did not yield the 5 and 6 nt products (Figure 9A, lane 4). These results demonstrate that WRN can readily stimulate FEN-1 cleavage of the 5 nt 5’ flap substrate.

FEN-1 has been shown to catalyze a 5’ to 3’ exonuclease reaction on nicked duplex DNA. A nick in the DNA duplex may arise at the site of a damaged base after incision by a DNA repair enzyme such as AP endonuclease. We tested the effect of WRN on FEN-1 cleavage activity at the site of the nick. FEN-1 (10 fmol) alone incised 2% of the nicked DNA duplex substrate molecules (Figure 9B, lane 2, and C). In the presence of WRN (75 fmol), FEN-1 incised 20% of the nicked DNA substrate (Figure 9B, lane 3, and C), a 10-fold stimulation of FEN-1 exonuclease activity. The stimulation of FEN-1 exonuclease activity by WRN was specific to the nicked DNA duplex; FEN-1 incision of ssDNA or blunt duplex double-stranded (ds)DNA was not stimulated by WRN.
Table 1. Sequence homology of RecQ proteins to the FEN-1 interaction domain WRN<sub>949–1092</sub>

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Sequence alignment of RecQ family members with the amino acid sequence 949–1092 of human WRN was performed using the GCG Lite Gap-Global Alignment of Two Sequences program (http://molfbio.info.nih.gov/molfbio/gcg-lite/compare.html), which utilized the BLOSUM62 amino acid substitution scoring matrix (Henikoff and Henikoff, 1992).

The ability of an immobilized recombinant C-terminal WRN fragment to precipitate nuclear FEN-1 suggested that this region of WRN might mediate the functional interaction with FEN-1. The results demonstrated that a 144-residue GST–WRN<sub>949–1092</sub> fragment was sufficient to specifically stimulate FEN-1 cleavage, indicating that this domain is responsible for the functional interaction with FEN-1. A sequence alignment of WRN<sub>949–1092</sub> with other RecQ family members indicated a number of highly conserved residues (Supplementary figure 2). The greatest degree of conservation was shared with FFA-1 (Table 1). Extensive sequence homology between FFA-1 and WRN exists throughout the open reading frame (Yan et al., 1998); therefore, it is not too surprising that these two proteins show such a high level of homology within the domain of WRN responsible for interacting with FEN-1. Nonetheless, the evident homology may point to a similar biochemical function of FFA-1 (or other RecQ family members) to stimulate FEN-1 cleavage. A role for FFA-1 in replication has been implicated (Yan and Newport, 1995; Chen et al., 2001). WRN (or FFA-1) may function with FEN-1 during replication to ensure efficient and accurate processing of specific DNA structures that arise during synthesis.

The identification of WRN in a replication complex (Lebel et al., 1999) suggests that WRN may play a direct role in replication. Like WS cells, rad27 mutants have increased spontaneous mutagenesis, genomic instability and impaired S phase progression (Reagan et al., 1995; Sommers et al., 1995; Vallen and Cross, 1995). The observation that rad27 temperature-sensitive mutants accumulate short DNA fragments of the expected size for unprocessed Okazaki fragments at the restrictive temperature suggests that FEN-1 participates in lagging strand synthesis in vivo (Merrill and Holm, 1998). WRN may function to stimulate FEN-1 incision of the remaining ribonucleotide, a step required prior to ligation of Okazaki fragments (Waga and Stillman, 1998). The ability of WRN to potently stimulate cleavage of the 1 nt 5' flap substrate and nicked DNA duplex supports this idea. WRN may also facilitate FEN-1 cleavage of longer 5' flap substrates that arise during strand displacement synthesis on the lagging strand. The 3' to 5' helicase activity of WRN and its ability to unwind RNA–DNA hybrids (Suzuki et al., 1997)

Discussion

We report here that WRN and FEN-1 physically interact and WRN stimulates FEN-1 cleavage by a direct protein interaction that is independent of energy and of WRN catalytic activity. The strong stimulation of FEN-1 cleavage by WRN was shown by the marked 82-fold increase in the rate of FEN-1 cleavage. Neither hPCNA nor hRPA displayed an effect on FEN-1 cleavage that approached the stimulation by WRN. Although the WRN interaction domain in FEN-1 is not yet known, it is possible that other structure-specific endonucleases related by sequence homology to FEN-1 (XPG and EXO1) are stimulated by WRN.

Fig. 9. WRN stimulates FEN-1 cleavage of duplex DNA substrates containing a longer 5' flap or a nick. Reactions (20 μl) containing 10 fmol of 5 nt 5' flap substrate (A) or a nicked duplex substrate (B) and 10 fmol of FEN-1 were incubated at 37°C for 15 min under standard conditions. The reactions in the presence of WRN contained 75 fmol of WRN. Substrate and cleavage products are as indicated. Lane 1, no enzyme; lane 2, FEN-1; lane 3, FEN-1 + wild-type WRN protein; lane 4, wild-type WRN protein. (C) % incision (mean value of three experiments) with SD for 5 nt flap and nicked duplex substrates is shown by gray and white bars, respectively.

(data not shown). We conclude that WRN specifically stimulates FEN-1 exonuclease activity on nicked duplex DNA.
suggests that WRN may translocate along the template strand and work in conjunction with a polymerase to displace the RNA primer (or DNA) of an Okazaki fragment. During replication restart, WRN may translocate to the stalled fork (Constantinou et al., 2000) and ensure efficient removal of the nascent lagging strand by its abilities to displace the nascent Okazaki fragment and stimulate the nucleases activities of FEN-1, similar to a model proposed for the coordinate action of *Escherichia coli* RecQ and RecJ (Courcelle and Hanawalt, 1999). This would prevent deleterious strand breakage or recombination to maintain genomic stability.

A role for WRN in NHEJ is suggested by its interaction with Ku (Cooper et al., 2000) and the hypersensitivity of WS cells to DNA cross-linking agents that induce dsDNA breaks (Poot et al., 2001). An *in vivo* role for FEN-1 in NHEJ has also been elucidated. Wu et al. (1999) demonstrated that a rad27 disruption mutant displayed a 4.4-fold reduction in the use of pathways that would require removal of short 2 nt 5‘ flaps. A model for NHEJ was presented in which FEN-1 removes the 5‘ flaps generated after end alignment prior to ligation. WRN may also participate in NHEJ or BER by regulation of patch size via its interaction with FEN-1. Future investigation of the importance of the WRN–FEN-1 interaction in DNA repair or replication should yield insight into the molecular defect responsible for genomic instability in WS.

Materials and methods

**Plasmid DNA constructions**

To construct GST–WRN plasmid constructs (Figure 1), PCR-amplified WRN DNA fragments from the human WRN gene kindly provided by Dr J.Oshima were subcloned into the bacterial expression plasmid pEXCS (Amersham) as described elsewhere (C.von Kobbe, P.Karmakar, L.Dawut, P.Opersko, X.Zeng, R.M.Brosh Jr, I.D.Hickson and V.A. Bohr, submitted).

**Proteins**

Histidine-tagged FEN-1, overexpressed in *E.coli* from a plasmid kindly provided by Dr M.Lieber, was purified on Ni2+-charged His-Bind resin (Novagen) as recommended by the manufacturer (Figure 1B). Recombinant His-tagged WRN protein (wild type, WRN-K577M, WRN-E84A) was overexpressed using a baculovirus/Sf9 insect system and purified as previously described (Orren et al., 1999) (Figure 1B). Drs M.Gray and J.Campisi kindly provided WRN-K577M and WRN-E84A baculovirus, respectively. Recombinant GST–WRN fusion proteins, overexpressed in *E.coli*, were purified on GS beads (Amerham Pharmacia Biotech) as recommended by the manufacturer (Figure 1B). A recombinant His-tagged WRN protein fragment corresponding to residues 940–1432 of the WRN sequence (His-WRN940-1432), overexpressed in *E.coli*, was purified as previously published (Cooper et al., 2000) (Figure 1B). hPCNA and hRPA were graciously provided by Dr M.Kenny (Figure 1B). Ku86/70 was kindly provided from Dr D.Ramsden.

**GST–WRN–Sepharose pull-down experiments**

GST–WRN fusion proteins were overexpressed in BL21(DE3) pLysS by 1 ml isopropyl-β-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, 0.4% Triton X-100]. The lysate was clarified by centrifugation at 35 000 r.p.m. (Ti 60 rotor; Beckman) for 1 h at 4°C. One milliliter of the resulting supernatant was incubated with 100 μl of GS beads (50% v/v) for 1 h at 4°C. The beads were washed three times with 1 ml of 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 750 μg of HeLa NE prepared as previously described (Dignam et al., 1983) or 1.2 μg of purified recombinant FEN-1 in 250 μl of buffer D (50 mM HEPES pH 7.1, 100 mM KCl, 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. Proteins were electrophoresed on 8% polyacrylamide SDS gels and transferred to PVDF membranes. Control membranes were stained with amido black reagent to demonstrate equal protein loading for samples. Membranes were probed with rabbit polyclonal anti-FEN-1 antibody (1:5000) followed by horse anti-rabbit IgG-horseradish peroxidase (HRP) (Vector) and ECL-Plus (Amersham Pharmacia).

**FEN-1–Sepharose pull-down experiments**

Purified FEN-1 or BSA (2 mg) was covalently coupled to 1 ml of cyanogen bromide-activated Sepharose (Amersham Pharmacia Biotech) as described by the supplier. FEN-1–Sepharose or BSA–Sepharose (25 μl) was washed three times with 300 μl of binding buffer (50 mM HEPES pH 7.9, 2 mM MgCl2, 0.02% Triton X-100, 0.1 mM EDTA) containing 10 mg/ml BSA. Washed beads were incubated for 1 h at 4°C with 25 μl of binding buffer containing 100 ng of WRN. Resin was washed three times with 300 μl of binding buffer, and then eluted with 20 μl of binding buffer containing 600 mM NaCl. Samples (10 μl) from the third wash or elution fractions were electrophoresed on a 10% polyacrylamide SDS gel and subsequently transferred to a nitrocellulose membrane. The membrane was probed by western blot analysis using rabbit anti-WRN antibodies (1:2000; Novus) and developed using goat anti-rabbit IgG-HRP (Vector) and ECL-Plus.

**WRN–FEN-1 co-immunoprecipitation experiments**

HeLa and AG11395 cells were grown in Dülbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum for 24 h at 37°C in 5% CO2. Cells (5 × 105) were washed in ice-cold PBS and lysed with 400 μl of IP buffer (1% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, 10 mM Tris pH 7.2, 1 mM EDTA) and Protease inhibitor Cocktail Set 2 (Calbiochem) at 4°C for 30 min. The suspension was centrifuged at 9000 g for 20 min at 4°C. The supernatant was incubated with goat polyclonal anti-WRN (1:40; Santa Cruz Biotech) for 2 h at 4°C and tumbled with 10 μl of protein G-agarose (Roche) at 4°C overnight. Beads were washed three times with IP buffer. Proteins were eluted by boiling treatment in Laemmli buffer, resolved on a 10% polyacrylamide Tris–glycine gel, and transferred to PVDF membrane. The membrane was probed using a mouse monoclonal anti-Fen-1 (1:100; Novocastra Labs) or anti-WRN antibody (1:250; BD PharMingen) followed by goat anti- mouse IgG-HRP (Amersham Pharmacia) and ECL-Plus.

**Oligonucleotide substrates**

PAGE-purified oligonucleotides (Midland Certified Reagent Co.) (Table II) were used for preparation of substrates. Downstream primers FLAP00, FLAP01 or FLAP05 were designed to form duplex substrates with a nick (Figure 8A), 1 nt 5‘ flap (Figure 3A) or 5 nt 5‘ flap (Figure 8A), respectively. Substrates were prepared as described previously (Tom et al., 2000).

| Table II. Oligonucleotide sequences for DNA substrates (5‘ to 3‘) |
|---|---|---|
| **Template** | **Length** | **Sequence** |
| TSTEM | 44 | GCAGTGGCGCTTCATTAAGGCTGT GACCTGGGAAACCCCTGGG |
| Downstream primer | FLAP00 | GTAAACGACGCGCCAGTGC |
| FLAP01 | 20 | AGTAAGACGACGACGCGAGTC |
| FLAP05 | 24 | TCCAAGTAAAAACGCGCGACGTC |
| Upstream primer | U25 | 25 | CGCCAGGGTTTCCCCAGTCAGCACG |
FEN-1 incision assay
Reactions (20 μl) contained 10 fmol of DNA substrate (except where indicated), and the indicated amounts of WRN and/or FEN-1 in 30 mM HEPES pH 7.6, 5% glycerol, 40 mM KCl (except where noted), 0.1 mg/ml BSA and 8 mM MgCl₂, WRN, hRPA, hPCNA or Ku80/70 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 (unless indicated otherwise), terminated with the addition of 10 μl of formamide dye (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 the ImageQuant software (Molecular Dynamics) was used for quantitation of the reaction products. Percentage incision was calculated from the equation: % incision = [(P(S + P)] × 100, where P is the sum of the intensity of the bands representing incision products, and S the intensity of the band representing the intact oligonucleotide. Background values from the no enzyme controls were subtracted out. In WRN controls that lacked FEN-1, the percentage of mononucleotide species was 0.5% or less.

Supplementary data
Supplementary data to this paper are available at The EMBO Journal Online.

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References


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