The Werner Syndrome Protein Promotes CAG/CTG Repeat Stability by Resolving Large (CAG)$_n$/(CTG)$_n$ Hairpins*

Received for publication, June 8, 2012, and in revised form, July 11, 2012. Published, JBC Papers in Press, July 11, 2012, DOI 10.1074/jbc.M112.389791

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Background: DNA hairpin repair (HPR) is an important genome maintenance system, but the components involved in HPR are unknown.

Results: WRN unwinds (CTG)$_n$ hairpins and facilitates HPR and polymerase δ-catalyzed DNA synthesis using a (CTG)$_{25}$ hairpin as a template.

Conclusion: WRN is involved in (CTG)$_n$ hairpin removal by resolving the hairpin, promoting DNA synthesis.

Significance: This work provides useful information to elucidate HPR and repeat instability-caused diseases.

Expansion of CAG/CTG repeats causes certain neurological and neurodegenerative disorders, and the formation and subsequent persistence of stable DNA hairpins within these repeats are believed to contribute to CAG/CTG repeat instability. Human cells possess a DNA hairpin repair (HPR) pathway, which removes various (CAG)$_n$ and (CTG)$_n$ hairpins in a nick-directed and strand-specific manner. Interestingly, this HPR system processes a (CTG)$_n$ hairpin on the template DNA strand much less efficiently than a (CAG)$_n$ hairpin on the same strand (Hou, C., Chan, N. L., Gu, L., and Li, G. M. (2009) Incision-dependent and error-free repair of (CAG)$_n$/(CTG)$_n$ hairpins in human cells. Nat. Struct. Mol. Biol. 16, 869–875), suggesting the involvement of an additional component for (CTG)$_n$ HPR. To identify this activity, a functional in vitro HPR assay was used to screen partially purified HeLa nuclear fractions for their ability to stimulate (CTG)$_n$ HPR. We demonstrate here that the stimulating activity is the Werner syndrome protein (WRN). Although WRN contains both a 3′ → 5′ helicase activity and a 3′ → 5′ exonuclease activity, the stimulating activity was found to be the helicase activity, as a WRN helicase mutant failed to enhance (CTG)$_n$ HPR. Consistently, WRN efficiently unwound large (CTG)$_n$ hairpins and promoted DNA polymerase δ-catalyzed DNA synthesis using a (CTG)$_n$ hairpin as a template. We, therefore, conclude that WRN stimulates (CTG)$_n$ HPR on the template DNA strand by resolving the hairpin so that it can be efficiently used as a template for repair or replicative synthesis.

CAG/CTG repeat instability is associated with certain neurological, neuromuscular, and neurodegenerative diseases, including Huntington disease, spinocerebellar ataxia, and myotonic dystrophy (1–4). These repeats can be located in either coding or noncoding regions of a gene. Once the expansion exceeds a threshold, it inactivates the expression of the affected gene or the function of the affected gene product, leading to the onset of diseases (3, 5).

Hairpin formation during DNA metabolism (e.g. replication and repair) is one of the favored mechanisms for triggering CAG/CTG repeat instability (6–9). Depending on whether the hairpin is formed on the nascent (i.e. newly synthesized or repaired) strand or the parental strand, DNA metabolic processing on CAG/CTG repeats can lead to repeat expansion or contraction, respectively. Recent studies have shown that human cells possess a hairpin repair (HPR) system that catalyzes error-free removal of CAG/CTG hairpins in a nick-dependent manner (10, 11). Regardless of the strand location of the CAG/CTG hairpins, the HPR system always targets the nicked (i.e. nascent) DNA strand for incisions, mainly using structure-specific endonucleases (11–13). If the hairpin is located in the nicked strand, the repair system removes the hairpin either by making dual incisions flanking the heterology or by a combination of nick-directed excision and flap endonucleolytic cleavage, which leaves a small single-strand gap. If the hairpin is located in the parental strand, incisions occur opposite the hairpin, followed by hairpin unwinding, which generates a relatively large single-strand gap. In either case, the gap is filled by replicative DNA polymerases using the continuous strand as a template (11). As a result, the HPR system ensures trinucleotide repeat (TNR) stability.

Interestingly, low repair efficiency was observed when a CTG hairpin was used as a template for resynthesis during hairpin repair (11). Because CTG repeats form a tighter hairpin than CAG repeats (14), it is hypothesized that the low repair efficiency of the CTG hairpin is because of polymerase impediement by the non-B DNA structure. Therefore, a CTG hairpin-
unwinding helicase should enhance the hairpin repair activity. Indeed, previous studies have implicated DNA helicases in maintaining TNR stability, presumably by resolving hairpins. Deletion of the Srs2 helicase from a yeast strain resulted in CAG/CTG repeat instability (15). In vitro studies reveal that Srs2 has high activity and specificity for unwinding CAG/CTG repeats (16). In addition, deletion of Sgs1 can cause repeat contraction when CTG is used as the template for lagging strand synthesis (17). Despite that the human homologs of Sgs1 (WRN and Bloom syndrome protein (BLM)) and Srs2 (proliferating cell nuclear antigen-interacting protein) have been identified (18–22), whether or not these helicases are involved in CAG/CTG hairpin repair remains unknown.

In this study, we provide evidence that the WRN is involved in (CTG)n hairpin repair on the template DNA strand. Using an in vitro HPR assay, we screened partially purified HeLa nuclear fractions for their ability to stimulate the repair of (CTG)n hairpins formed in the template strand. This analysis identified the WRN helicase as such a stimulating factor. A primer extension assay showed that WRN could stimulate polymerase δ-catalyzed DNA synthesis on a (CTG)n template, whereas a helicase assay revealed that WRN could resolve (CTG)n hairpins. These results, therefore, suggest that WRN contributes to CAG/CTG repeat stability by resolving (CTG)n hairpins during DNA synthesis.

**EXPERIMENTAL PROCEDURES**

**Preparations of HeLa Nuclear Extract and Recombinant Proteins**—HeLa S3 cells were cultured to a density of 5 × 10⁵ cells/ml in RPMI 1640 medium supplemented with 5% fetal bovine serum and harvested for nuclear extract preparation as described (23). DNA polymerase δ (pol δ), replication factor C, and WRN were expressed in High Five insect cells using the baculovirus system, and proliferating cell nuclear antigen and replication protein A were expressed in *Escherichia coli*. These recombinant proteins were purified essentially as described (24–27). All the purified proteins were at least 95% homogeneous as visualized on Coomassie Blue-stained SDS-polyacrylamide gels. Protein concentrations were determined by the BioRad protein assay kit using BSA as a standard. The expression vectors for pol δ, WRN, proliferating cell nuclear antigen, and replication protein A were gracious gifts from Drs. Ellen Fanning (Vanderbilt University), Larry Loeb (University of Washington), Jerard Hurwitz (Memorial Sloan-Kettering Cancer Center), and Marc Wold (University of Iowa), respectively.

**Phosphocellulose Chromatography of HeLa Nuclear Extracts**—Phosphocellulose P-11 fractions were prepared as described in Ref. 28. Briefly, HeLa nuclear extract (300 mg) was diluted to 5 mg/ml with buffer A (25 mM HEPES-KOH, pH 7.5, 0.1 mM EDTA, 1× protease inhibitor containing 100 mM PMSF, 191.5 mM benzamidine, 0.05 g/liter pepstatin A, 0.05 g/liter leupeptin). Diluted extract was loaded onto a phosphocellulose column (Whatman P-11, 6 cm × 10 cm) equilibrated with buffer A containing 50 mM KCl. After washing with 200 ml of the same buffer, the column was eluted with a 0.8-liter linear gradient of 0.05–1.3 M KCl in buffer A. Fractions of 6 ml were collected for a total of 130 fractions and dialyzed against 1 liter of buffer A containing 100 mM KCl. Finally, the individual fractions were aliquoted, frozen in liquid nitrogen, and stored at −80 °C.

**DNA Hairpin Substrate and Hairpin Repair Assay**—A circular plasmid DNA substrate (Fig. 1), containing a (CTG)₂₅ hairpin in the continuous strand and a nick 149-bp 5’ to the hairpin, was constructed by hybridizing single-stranded M13mp18-(CTG)₃₅ phage DNA with BglII-linearized double-stranded M13mp18-(CAG)₁₀ DNA as described (11). Unless mentioned otherwise, hairpin repair was assayed as described (11) in a 40-μl reaction containing 200 ng (42 fmol) of the DNA hairpin substrate, 50 or 100 μg of HeLa nuclear extract in the presence or absence of partially purified HeLa nuclear fractions or purified WRN, as indicated. DNA was recovered by sequential phenol extraction and ethanol precipitation and digested with BsrBI and BglII (see Fig. 1). The resulting DNA products were separated on a 6% denaturing polyacrylamide gel and subjected to Southern blotting analysis using a 32P-labeled oligonucleotide probe specifically annealing to the smaller fragment of the BsrBI-BglII digestion in the nicked strand (see Fig. 1, green bar) to score for conversion of 10 CAG/CTG repeats to 35 CAG/CTG repeats. Repair products, as well as unrepaired molecules, were visualized by exposing to an x-ray film or a Phosphor-
RESULTS

A Partially Purified HeLa Nuclear Fraction Containing WRN Stimulates (CTG)$_n$ HPR—To identify protein factors involved in the repair of CTG hairpins formed in the template (continuous) DNA strand, we screened partially purified HeLa nuclear activities chromatographed on a phosphocellulose P-11 column (28, 29) for their ability to stimulate the removal of a (CTG)$_{35}$ hairpin using an in vitro HPR assay (Fig. 1). The results showed that P-11 fractions 68–76 greatly stimulated the (CTG)$_{35}$ HPR, as judged by the fact that adding these fractions to 50 μg of HeLa nuclear extract increased the repair from ~16 to 26% (Fig. 2, A and B), contributing >38% (i.e. 10/26) in the overall repair. The stimulation is not simply because of the addition of an extra amount of proteins to the reaction, as fraction 76 contains less amount of WRN than fractions 68 and 72, it stimulated the repair reaction at a level similar to that of fractions 68 and 72 (Fig. 2, A and B). We believe that this is likely because of the fact that this fraction also contains pol δ and pol ε (Fig. 2C), which have been postulated to be involved in HPR (10, 11).

WRN Stimulates pol δ-catalyzed DNA Synthesis on a (CTG)$_{35}$ Template—It has been shown that WRN interacts with pol δ and rescues pol δ-mediated replication at forks stalled by unusual DNA secondary structures including (CGG)$_n$-formed hairpins (30). We, therefore, believe that WRN stimulates the (CTG)$_{35}$ hairpin repair by promoting pol δ-catalyzed DNA synthesis using a (CTG)$_h$ hairpin as a template. To test this hypothesis, we conducted pol δ-catalyzed primer extension in the presence of purified WRN using a (CTG)$_{35}$-formed hairpin as a template (Fig. 3A). The result shows that WRN indeed stimulates DNA synthesis on the (CTG)$_{35}$-containing template (Fig. 3B, left gel). Because WRN belongs to the RecQ helicase family (31), two other members of this family, RecQ1 and BLM, were also tested for their role in stimulating DNA synthesis using the same CTG hairpin-containing template. However, unlike WRN, neither BLM (data not shown) nor RecQ1 could stimulate the DNA synthesis on the (CTG)$_{35}$ template (Fig. 3B, right gel). Consistent with this result, HeLa extract-catalyzed HPR was not stimulated by the addition of purified BLM or RecQ1 (data not shown). These observations suggest that WRN is more specific for repeat-formed hairpin structures than other RecQ family members. To confirm further that the stimulating activity is specifically related to WRN, a WRN mutant (K577M)
(CAG)_n/(CTG)_n Hairpin Unwinding by WRN

**FIGURE 3.** WRN enhances pol δ-catalyzed DNA synthesis. A, diagram of the primer extension assay. The (CTG)_35-containing circular M13mp18 ssDNA was linearized with restriction enzymes BstNI and BsrBI after annealing with oligonucleotides complementary to the corresponding restriction sequences. A 5′-32P-labeled primer was then annealed with the M13mp18 template for DNA synthesis. B, pol δ-catalyzed primer extension using a (CTG)_35 hairpin as a template in the presence of various amounts of purified WRN or RecQ1, as indicated. C, pol δ-catalyzed primer extension using a (CTG)_35 hairpin as a template in the presence of wild-type WRN (W) or a helicase-deficient WRN mutant (M), as indicated. Reaction products were resolved in 6% denaturing polyacrylamide gels and visualized by a PhosphorImager. The total synthesis includes the product intermediates.

defective in the helicase activity (24, 32) was used in the primer extension analysis. As shown in Fig. 3C, wild-type (W) WRN but not the K577M mutant (M) enhanced pol δ-catalyzed DNA synthesis on the (CTG)_35 template, implying involvement of the WRN helicase activity in stimulating DNA synthesis on a hairpin template.

WRN Resolves (CTG)_n Hairpins—To determine whether WRN promotes (CTG)_n hairpin repair by resolving the hairpin structure in the template strand, we designed a helicase assay to detect CTG hairpin unwinding (Fig. 4A). To ensure a high percentage hairpin formation, a 32P-labeled oligonucleotide containing 35 CTG repeats was heated and then slowly cooled down to room temperature. The resulting hairpin structure was incubated with a complementary oligonucleotide containing 35 CTG repeats and slowly cooled down to ensure intra-strand hairpin formation. The hairpin substrate was incubated with purified WRN protein, followed by incubation with an unlabeled (CAG)_35-containing oligonucleotide, respectively, allowing for the formation of a dsDNA product with 5′ overhangs that is refractory to WRN unwinding and migrates differently from ssDNA in nondenaturing polyacrylamide gels. B, (CTG)_35 hairpin unwinding by WRN or the K577M helicase mutant and its annealing with a (CAG)_35 oligonucleotide in the presence or absence of ATP, as indicated. C, (CTG)_35 hairpin unwinding by WRN or the K577M helicase mutant and its annealing with a (CAG)_35 oligonucleotide in the presence of ATP. The unwinding-reannealing products were analyzed by nondenaturing polyacrylamide gel electrophoresis and detected by a PhosphorImager.

**FIGURE 4.** WRN resolves CTG hairpins. A, diagram of the unwinding-reannealing assay. S′ 32P-labeled (CTG)_15 or (CTG)_35 oligonucleotide was heated and slowly cooled down to ensure intra-strand hairpin formation. The hairpin substrate was incubated with purified WRN protein, followed by incubation with an unlabeled (CAG)_15 or (CAG)_35-containing oligonucleotide, respectively, allowing for the formation of a dsDNA product with 5′ overhangs that is refractory to WRN unwinding and migrates differently from ssDNA in nondenaturing polyacrylamide gels. B, (CTG)_35 hairpin unwinding by WRN or the K577M helicase mutant and its annealing with a (CAG)_35 oligonucleotide in the presence or absence of ATP, as indicated. C, (CTG)_35 hairpin unwinding by WRN or the K577M helicase mutant and its annealing with a (CAG)_35 oligonucleotide in the presence of ATP. The unwinding-reannealing products were analyzed by nondenaturing polyacrylamide gel electrophoresis and detected by a PhosphorImager.

**4B, lanes 9–12.** This result is consistent with the fact that ATP is required for WRN helicase activity (24). In addition, substitution of wild-type WRN with the K577M helicase mutant prevented the generation of the dsDNA product (Fig. 4B, lanes 13–16), confirming further that WRN unwinds CTG hairpins using its helicase activity. Similar results were obtained when a (CTG)_15 hairpin substrate was used in this analysis (Fig. 4C). However, it appears that more dsDNA products were generated in reactions with 15 CTG/CAG repeats than those with 35 CTG/CAG repeats (compare Fig. 4B with Fig. 4C), indicating that a smaller hairpin is more easily resolved than a larger hairpin.

It is worth mentioning that the data shown in Fig. 4 also suggest that WRN unwinds CAG hairpins. It is known that single-stranded CAG or CTG repeats can form hairpin structures spontaneously (6, 9). Thus, like the (CTG)_35-containing substrate, the (CAG)_35-containing oligonucleotide also carries a hairpin. If the CAG hairpin is not resolved, the CTG repeats will not be able to anneal with the CAG repeats. The observation of the dsDNA formation indicates that both the CTG and CAG hairpins have been resolved by WRN. However, because a CTG hairpin is more stable than a CAG hairpin (9, 14), its unwinding requires more enzymatic activities, explaining why the CTG HPR activity is much weaker than the CAG HPR activity in HEK293 extracts (11). Consistent with this assumption, we show here that the CTG hairpin repair can be greatly enhanced by the addition of exogenous WRN protein. Therefore, our data strongly suggest that WRN helicase promotes HPR by unwinding...
ing both the CTG and CAG hairpin structures so that they can be effectively used as a template for DNA synthesis.

**DISCUSSION**

WRN, a member of the RecQ helicase family, plays an important role in DNA metabolic pathways, including DNA replication, recombination, repair, and telomere maintenance (31, 34). WRN possesses a helicase activity that unwinds dsDNA in a 3’→5’ directionality (35). The loss of WRN function results in replication abnormalities, chromosomal instability, cancer predisposition, and premature aging (36). One of the postulated roles for WRN is preventing replication fork collapse, possibly by using its helicase activity to resolve non-B DNA structures that interfere with replication fork progression (37). We demonstrate here that the WRN helicase facilitates large (CTG)$_n$ HPR via unwinding the hairpin structure in the template DNA strand, thereby promoting pol δ-catalyzed DNA synthesis.

Previous studies have shown that human nuclear extracts possess a HPR pathway that is capable of processing a variety of (CAG)$_n$//(CTG)$_n$ hairpins in an error-free and nick-dependent manner (10, 11, 13). The HPR system targets the nicked (i.e. nascent) DNA strand for incisions using structure-specific endonucleases (11–13). Interestingly, the system repairs a (CTG)$_n$ hairpin with much less efficiency than a (CAG)$_n$ hairpin when they are on the template DNA strand (11). Using a functional in vitro assay, we identified an activity from HeLa nuclear extracts that stimulates (CTG)$_n$ HPR, and this activity was confirmed to be the WRN helicase (Fig. 2). This finding is consistent with the following observations. First, a CTG repeat-formed hairpin is more stable than a CAG repeat-formed hairpin (9, 14) and would require more efforts to resolve it before it would be used as a template for DNA synthesis. Second, failure to resolve a (CTG)$_n$ hairpin in the template DNA strand would lead to contractions of the CTG repeats. Thus, deletions that occur prominently in WRN-deficient cells (38) might be due in part to an inability to properly resolve hairpins and other secondary structures. In agreement with this general concept, WRN-deficient cells show more frequent chromosomal breakage for at least several fragile sites (39), one of which contains dinucleotide repeats with the potential to form hairpin structures. Our results suggest that this increased fragility could be because of endonuclease-catalyzed incisions at hairpin structures in the absence of WRN. Third, the yeast Srs2 and Sgs1 helicases have been shown to stabilize CAG/CTG repeats (15, 16), particularly to prevent CTG repeats in the template strand from contraction (17), suggesting that these yeast helicases also unwind (CTG)$_n$ hairpins during DNA synthesis. However, it is interesting to note that although both WRN and BLM are the human homologs of Sgs1 (19), it is WRN but not BLM that is capable of stimulating (CTG)$_n$ HPR (Fig. 3 and data not shown). These observations suggest that WRN has a substrate specificity for (CTG)$_n$//(CAG)$_n$ hairpins.

We attribute the WRN-stimulating activity for (CTG)$_n$ HPR to its helicase activity. This assumption is based on the fact that (i) WT WRN, but not the helicase-deficient K577M WRN, can resolve (CTG)$_{35^\text{n}}$ or (CTG)$_{15^\text{n}}$-formed hairpins and promote pol δ-catalyzed DNA synthesis (Fig. 3); (ii) in the helicase assay, the production of dsDNA occurs only in the presence of ATP (Fig. 4), which is essential for the WRN helicase activity (35); and (iii) the WRN helicase mutant (K577M) fails to generate dsDNA products (Fig. 4).

Taken together, our results support a model depicted in Fig. 5. Upon incision(s) opposite the CTG hairpin by an endonuclease required for DNA HPR, pol δ is recruited to conduct repair DNA synthesis. However, the CTG hairpin in the template DNA strand causes pol δ to pause at the non-B DNA structure. To effectively remove the hairpin, WRN, which physically interacts with pol δ (30, 40, 41), is recruited to the site to resolve the CTG hairpin, allowing pol δ to carry out the repair DNA synthesis using the unstructured CTG repeats as a template. In addition to DNA HPR, this model also applies to DNA replication, where a CTG hairpin can be formed in either the template strand or the primer strand, particularly the pol δ-catalyzed lagging strand DNA synthesis (42, 43) because of the availability of free DNA ends in the Okazaki fragments and single-strand DNA regions in the lagging template. The hairpin unwinding by WRN in each case is essential for preventing repeat instability: i.e. expansion when the hairpin occurs in the newly synthesized strand and contraction when it is formed in the template strand. Therefore, WRN plays an important role in maintaining TNR stability during DNA replication and repair by resolving CTG hairpins. This finding provides useful information for understanding the TNR expansion-caused diseases, as well as the molecular mechanism of the HPR pathway.

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5. Acknowledgments—We thank Drs. Ellen Fanning, Larry Loeb, Jerard Hurwitz, and Marc Wold for reagents, and Christopher Pearson and Charles Ensor for comments on the manuscript.

![Diagram of (CAG)$_n$//(CTG)$_n$ Hairpin Unwinding by WRN](image-url)
(CAG)m/(CTG)n Hairpin Unwinding by WRN

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