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DNA secondary structure of the released strand stimulates WRN helicase action on forked duplexes without coordinate action of WRN exonuclease

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ABSTRACT

Werner syndrome (WS) is an autosomal recessive premature aging disorder characterized by aging-related phenotypes and genomic instability. WS is caused by mutations in a gene encoding a nuclear protein, Werner syndrome protein (WRN), a member of the RecQ helicase family, that interestingly possesses both helicase and exonuclease activities. Previous studies have shown that the two activities act in concert on a single substrate. We investigated the effect of a DNA secondary structure on the two WRN activities and found that a DNA secondary structure of the displaced strand during unwinding stimulates WRN helicase without coordinate action of WRN exonuclease. These results imply that WRN helicase and exonuclease activities can act independently, and we propose that the uncoordinated action may be relevant to the *in vivo* activity of WRN.

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1. Introduction

Werner syndrome (WS) is a human autosomal recessive disorder caused by mutations in the *WRN* gene [1] and characterized by premature aging and increased incidence of cancer [2]. WS cells show genome instability, such as various types of chromosomal aberrations [3], accelerated replicative senescence [4], and accelerated telomere loss [5]. In addition, WS cells are sensitive to many different types of DNA damaging agents, such as 4-nitroquinoline 1-oxide (4-NQO), camptothecin (CPT), and inter-strand crosslinks (ICLs) [6–8].

WRN is a member of the RecQ helicase family and has both helicase and exonuclease activities [9,10]. WRN helicase unwinds a variety of DNA structures [11,12]. WRN exonuclease degrades nucleotides from 3′-recessed termini, gaps, nicks, and blunt ends of forked duplexes [12,13]. The two activities of WRN are modulated by interacting proteins that are involved in DNA repair, replication, and/or recombination pathways [14–17], thus suggesting that WRN plays an important role in these DNA transactions.

WRN helicase and exonuclease can simultaneously act on a single forked DNA duplex [13]. The WRN helicase unwinds at the fork of the substrate, while the WRN exonuclease digests at the blunt end. This coordinated action results in removing a DNA strand from the long, forked duplex that is not completely unwound by WRN helicase.

However, WRN can completely unwind 16- and 22-bp forked duplexes without digestion of the duplexes. A rapid unwinding of the short forked duplexes coincides with a lack of exonuclease progression with time. In addition, in the presence of RPA, WRN can unwind a 34-bp forked duplex and release the full-length strand without shortening the forked duplex [13]. RPA stimulation of WRN helicase thereby inhibits WRN exonuclease on the forked duplex and WRN helicase may act independently of WRN exonuclease.

Although the exact mechanism for the regulation of WRN exonuclease is not understood, one question raised by these studies is whether the stability of the remaining duplex during unwinding and the displaced strand can regulate WRN helicase and exonuclease activities.

In this study, we aimed to identify DNA structural elements that influence WRN activities. Our results herein reveal that a DNA secondary conformation, such as a hairpin loop of the displaced strand, leads WRN to unwind forked duplexes proficiently and that the location of the secondary structure with respect to the fork affects WRN helicase efficiency. Thus, this study suggests that WRN helicase can unwind forked duplexes without coordinating with the WRN exonuclease.

2. Materials and methods

2.1. Proteins and oligonucleotide substrates

Oligonucleotides were ordered from Integrated DNA Technologies (Coralville, IA, USA). For biochemical assays (see below), the indicated oligonucleotides in figures were radiolabeled at the 5′

Abbreviations: RPA, replication protein A; WRN, Werner syndrome protein; WS, Werner syndrome; dsDNA, double-stranded DNA.

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end (5′ end-labeled) with [γ-^{32}P] ATP (3000 Ci/mmol, Amersham Radiochemicals) using T4 polynucleotide kinase (New England Biolabs) for 30 min at 37 °C and heat-inactivated for 10 min at 95 °C. Forked duplex substrates were formed by annealing the labeled oligonucleotides to their unlabeled complementary strands at a molar ratio of 1:2 by incubation, as previously described [13].

Recombinant wild-type WRN and X-WRN containing a point mutation (E84A) in the exonuclease domain were expressed using a baculovirus/insect cell expression system and purified, as described previously [18]. The recombinant glutathione *S*-transferase (GST)-tagged WRN fragment, HlcRQC, was purified, as described previously [19]. Protein concentrations were determined by a Bio-RAD assay (BIO-RAD) using BSA as the standard.

2.2. WRN helicase and exonuclease reactions

DNA substrates were mixed in reaction buffer (10–20 µl, 40 mM Tris–Cl, pH 8.0, 4 mM $MgCl_2$, 5 mM DTT, 2 mM ATP, and 0.1 mg/ml BSA) or 'exo-free' buffer (10–20 µl, 50 mM Hepes–KOH, pH 7.5, 2 mM $MgCl_2$, 5 mM DTT, 2 mM ATP, and 0.1 mg/ml BSA). In all cases, the concentration of DNA substrates was 1.0 nM. Reactions were initiated by the addition of proteins to the reaction mixture and incubated for 15 min at 37 °C.

2.3. Analysis of helicase and exonuclease products

Reactions were terminated by the addition of 3× stop dye (50 mM EDTA, 40% glycerol, 0.9% SDS, 0.1% bromophenol blue, and 0.1% xylene cyanol) to a 1× final concentration, along with a 100-fold molar excess of an unlabeled competitor oligonucleotide (identical to the labeled oligonucleotide strand of forked duplexes) to prevent reannealing of the unwound-radiolabeled ssDNA products. Helicase products were analyzed on native polyacrylamide gels, and visualized using a PhosphorImager (Typhoon 9400, GE Life Science).

Reactions were terminated by an addition of equal volume of formamide stop dye (80% formamide, 0.5× Tris borate, 0.1% bromophenol blue, and 0.1% xylene cyanol). Reaction products were heat-denatured for 5 min at 95 °C, analyzed on 14% sequencing polyacrylamide gels, and visualized using a PhosphorImager.

2.4. DNA secondary structure prediction

The potential secondary structure for all DNA oligonucleotides was found using the structure prediction program, RNAfold, at rna.tbi.unive.ac.at/cgi-bin/RNAfold.cgi (web-based program). Structure predictions were determined using DNA parameters and the default options in the program.

2.5. Native gel electrophoresis

Single-stranded DNA oligonucleotides were 5′-radiolabeled, as described in the oligonucleotide substrates above. DNA oligonucleotides were then incubated for 15 min at 37 °C in buffer plus 10% glycerol. DNA oligonucleotides (1 pmol) were analyzed on a 10% nondenaturing polyacrylamide gel (19:1 acrylamide/bis) and electrophoresed at 160 v in 1× TBE for 2.5 h at room temperature. Radiolabeled DNA species were visualized using a PhosphorImager.

3. Results

3.1. Sequence of the DNA substrates affects WRN enzymatic activities

A WRN digestion analysis of a forked duplex containing 4 telomeric repeats (TTAGGG) noticeably showed short products of 39-, 32-, and 26 nt-long fragments (Fig. 1A) as shown in a previous report [13]. The corresponding DNA bases to these fragments were either at the end of or within a homopolymeric run of three 3Gs (GGG in the 34-bp forked duplex, labeled as 3G), as shown in Fig. S1. This observation suggests that WRN exonuclease activity may be affected by DNA sequence context.

To examine the influence of the DNA sequence context on WRN exonuclease and helicase activities, we replaced GGG in the 34-bp forked duplex with GGGG (labeled as 4G) or CGCG (2CG), as shown in Fig. S1, and the three DNA substrates were incubated with increasing amounts of WRN. Reaction products were analyzed on native gels to show WRN helicase activity and on denaturing gels to visualize WRN exonuclease activity.

An analysis of the reaction products of the 4G substrate on a denaturing gel revealed that WRN degraded the 5′-labeled strands of the 4G substrate, starting from the blunt end (Fig. 1A). The pattern of digestion of the 4G substrate showed prominent bands of 38 and 31 nt in length (Fig. S1). In the 2CG substrate, prominent bands longer than 37 nt were predominantly detected, and the extent of digestion of the full-length 49-mer strand was less than that

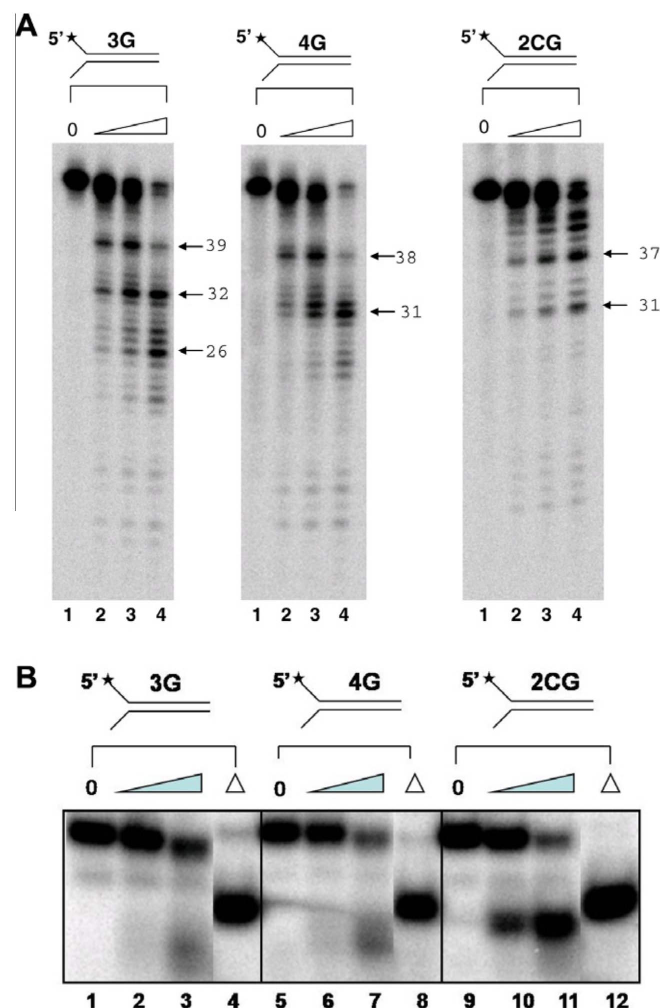

Fig. 1. DNA sequences on forked duplexes influence both WRN exonuclease and helicase activities. WRN protein was incubated with the 34-bp forked duplexes (1.0 nM) under the standard reaction conditions at 37 °C for 15 min. Reactions were terminated in the appropriate stop dye. (A) Analysis of wild-type WRN exonuclease activity. DNA substrates and 0 (lane 1), 1.7, 3.4, 6.7 ng WRN (lanes 2–4). Reaction products were run on a 14% denaturing polyacrylamide gel. Values indicate the product length and extent of degradation. (B) Analysis of wild-type WRN helicase activity. DNA substrates and 0 (lane 1), 1.7 and 3.4 ng WRN (lanes 2 and 3). Reaction products were run on a 12% native polyacrylamide gel. Δ, heat-denatured substrate control (lane 4).

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