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# Conserved helicase domain of human RecQ4 is required for strand annealing-independent DNA unwinding

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ABSTRACT

Humans have five members of the well conserved RecQ helicase family: RecQ1, Bloom syndrome protein (BLM), Werner syndrome protein (WRN), RecQ4, and RecQ5, which are all known for their roles in maintaining genome stability. BLM, WRN, and RecQ4 are associated with premature aging and cancer predisposition. Of the three, RecQ4's biological and cellular roles have been least thoroughly characterized. Here we tested the helicase activity of purified human RecQ4 on various substrates. Consistent with recent results, we detected ATP-dependent RecQ4 unwinding of forked duplexes. However, our results provide the first evidence that human RecQ4's unwinding is independent of strand annealing, and that it does not require the presence of excess ssDNA. Moreover, we demonstrate that a point mutation of the conserved lysine in the Walker A motif abolished helicase activity, implying that not the N-terminal portion, but the helicase domain is solely responsible for the enzyme's unwinding activity. In addition, we demonstrate a novel stimulation of RecQ4's helicase activity by replication protein A, similar to that of RecQ1, BLM, WRN, and RecQ5. Together, these data indicate that specific biochemical activities and protein partners of RecQ4 are conserved with those of the other RecQ helicases.

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## 1. Introduction

The RecQ family represents a group of helicases well conserved from bacteria to humans. Unlike bacteria and yeast, which have only one family member, humans have five distinct helicases: RecQ1, Bloom syndrome protein (BLM), Werner syndrome protein (WRN), RecQ4, and RecQ5. To date, three of these, BLM, WRN, and RecQ4, have been linked to premature aging and cancer predisposition. While the roles of BLM and WRN in DNA repair, DNA replication, and telomere maintenance have been characterized extensively, relatively little is known about the biological and cellular functions of RecQ4 [1–5].

RecQ4 deficiencies have been linked to three rare autosomal recessive diseases—Baller–Gerold syndrome, RAPADILINO syndrome, and Rothmund–Thomson syndrome (RTS). RTS symptoms include developmental abnormalities, growth deficiencies, proneness to cancer, predominantly osteosarcomas, and premature aging, including development of cataracts and hair loss [6,7]. Cells from RTS patients display chromosomal instability and aneuploidy [7–11], in addition to sensitivity to replication inhibitors and oxidative stress [12,13]. Although RTS is not exclusively caused by defects in the *RECQ4* gene, a majority of RTS patients have mutations in *RECQ4*, most within the conserved helicase domain [14–16]. Together these results are indicative of a role for RecQ4 in processes that maintain genome stability and suggest that the helicase activity is important for RecQ4's biological and cellular roles.

More recently the cellular roles of RecQ4 in DNA replication and repair have been explored. Studies of the RecQ4 homolog in *Xenopus* revealed that it is important for loading replication factors at origins of replication [17]. Consistent with this work, subsequent reports showed that *Xenopus* RecQ4 promotes association of polymerase α with chromatin during replication initiation [18].

Additionally, *Xenopus* RecQ4 is loaded in a replication-independent manner onto chromatin containing double strand breaks, suggesting a role for RecQ4 in DNA repair processes, as well [19]. *Drosophila RECQ4* mutants display sensitivity to gamma irradiation, along with deficiency in repair of double strand breaks *in vivo*, in support of RecQ4 participation in double strand break repair [20]. Moreover, following treatment with the double strand break-inducing agent etoposide, RecQ4 co-localized with both Rad51 foci and regions of single-stranded DNA (ssDNA), implying its involvement in the homologous recombination pathway of double strand break repair [21].

*Abbreviations:* APE1, apurinic endonuclease 1; BER, base excision repair; BLM, Bloom syndrome protein; bp, base pair; FEN1, flap endonuclease 1; NER, nucleotide excision repair; nt, nucleotide; RPA, replication protein A; RTS, Rothmund–Thomson syndrome; SFII, superfamily II; ssDNA, single-stranded DNA; WT, wild-type; WRN, Werner syndrome protein.

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RecQ4 participation in DNA repair also extends to other pathways. A recent study demonstrated that, following UV irradiation, RecQ4 co-localized and interacted directly with xeroderma pigmentosum group A protein, which mediates the associations of repair machinery around damaged DNA during nucleotide excision repair (NER) [22]. In addition, roles of RecQ4 in base excision repair (BER) have been recently discovered. RecQ4 physically interacts with poly-(ADP ribose) polymerase-1, which has an important role in regulation of BER through (ADP-ribosyl)ation of component proteins [23]. RecQ4 stimulation of key players in BER, namely DNA polymerase β, apurinic endonuclease 1 (APE1), and flap endonuclease 1 (FEN1) is evidence of a direct functional involvement of RecQ4 in BER, as well [24]. Furthermore, studies with RTS cells showed increased formation of strand breaks upon exposure to hydrogen peroxide and accumulation of XRCC1 foci following oxidative stress [24]. Also, treatment with the single strand break-inducing agent paraquat resulted in decreased survival in *Drosophila RECQ4* mutants [20]. Together these results signify a role of RecQ4 in single strand break repair.

Although the precise mechanisms of RecQ4 functions in these cellular replication and repair processes have yet to be elucidated, they are likely linked to its biochemical activities. Generally, the RecQ helicases RecQ1, BLM, WRN, and RecQ5 have similar biochemical activities, including 3′–5′ ATP-dependent DNA unwinding and strand annealing [4]. Initial biochemical characterization of RecQ4 demonstrated ATPase and strand annealing activities without detectable unwinding activity [25,26]. However, in recent studies, purified human RecQ4 displayed helicase activity [27,28]. Xu and Liu proposed that the helicase activity of RecQ4 is relatively weak compared to its strand annealing activity, which regenerates the native substrate following unwinding. RecQ4's helicase activity was only revealed when excess of ssDNA was used to trap the released strand [28]. Based on its homology to the other RecQ helicases and on the conserved strand annealing and ATPase activities, it is not surprising that RecQ4 would also display helicase activity. Yet, the unwinding activity of RecQ4 has not been fully characterized. In the current report we set out to further analyze the helicase activity of purified human RecQ4 through studies *in vitro*.

## 2. Materials and methods

### 2.1. Enzyme expression and purification

Wild-type (WT) human RecQ4 with a C-terminal 9-histidine tag in the pGEX6p1 vector (GE Healthcare) was expressed and purified from *E. coli* Rosetta2 (DE3) (Novagen) as described previously, with the following modifications [25]. Cells were lysed by sonication pulses 30 s on then 30 s off for a total of 7 min at 50% power in lysis buffer containing 50 mM Tris–HCl pH 7.5, 200 mM KCl, 10% sucrose, 2 mM EDTA, 1 mM DTT, 0.01% Igepal (Sigma), and 5 μg/ml each of aprotinin, chymostatin, leupeptin, and pepstatin A protease inhibitors. The extract was clarified by low speed centrifugation at 8000 rpm (rotor JA-12, Beckman Coulter) for 15 min followed by ultracentrifugation at 40,000 rpm (rotor 60 Ti, Beckman Coulter) for 30 min. Lysate was passed through a 70 ml Q Sepharose column (GE Healthcare) and then onto a 40 ml SP Sepharose column (GE Healthcare). Protein was eluted by a gradient of 200–660 mM KCl in K Buffer (20 mM $KH_2PO_4$, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, and 1 mM DTT). The glutathione–sepharose fast flow matrix (GE Healthcare) was washed with three times 30 ml K buffer containing 500 mM KCl. Following elution from the nickel-NTA agarose (Novagen) with 300 mM imidazole in K buffer containing 500 mM KCl, the RecQ4 was identified by electrophoresis and Coomassie Blue staining of a polyacrylamide gel. The RecQ4 was pooled, dialyzed in K buffer containing 500 mM KCl overnight at 4 °C to remove the imidazole, aliquoted, and stored at −80 °C.

Human RecQ4 mutant K508 M (KM) was generated by PCR amplification of the WT RecQ4 construct using forward and reverse primers 5′-ATGTCCCTGTGCTACCAGCTC-3′ and 5′-GCCGGCACCTGTAGGCAGCAC-3′, respectively. PCR product was ligated and amplified in bacteria. Final sequence-verified construct was expressed in *E. coli* Rosetta2 (DE3) (Novagen). Expression and purification of the KM RecQ4 was as described above for WT. Final fractions of both WT and KM RecQ4 were analyzed by SDS-PAGE and Coomassie Blue gel staining. Human RPA was expressed from plasmid vector p11d-tRPA (gift from Dr. Marc S. Wold) [29] in *E. coli* BL21 (Novagen) and purified as previously described [30]. Human WRN was expressed and purified using a baculovirus/Sf9 insect cell system as previously described [31].

### 2.2. Oligonucleotide substrates

Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA), and sequences are listed in Table 1 and Supplementary Table 1. Primers $T_{1-9}$, $HJ_1$, and $G_1$ were radiolabeled at the 5′ end with [γ-$^{32}$P] ATP (PerkinElmer Life Sciences) and T4 polynucleotide kinase (New England Biolabs). Following labeling, unincorporated [γ-$^{32}$P] ATP was removed from $T_{1-9}$ using MicroSpin G-25 columns (GE Healthcare). Then to anneal the fork and full duplex substrates, corresponding oligonucleotides were combined in annealing buffer (40 mM Tris–HCl pH 8.0 and 50 mM NaCl) in a 1:2 ratio (labeled to unlabeled oligonucleotide), heated at 95 °C for 5 min, and cooled gradually to room temperature. The Holliday junction [32,33], and G-quadruplex [34,35] substrates were prepared as previously described. Substrates used for individual experiments are indicated in the figure legends and depicted in each figure.

### 2.3. Enzyme assays

All assays were performed at least in triplicate. For helicase assays, RecQ4 (amount indicated in figure legend) was incubated with substrate (0.5 nM) for 30 min at 37 °C in 10 μl helicase buffer containing 30 mM Tris pH 7.4, 5 mM $MgCl_2$, 1 mM DTT, 100 μg/ml

**Table 1**
Oligonucleotide sequences.

| Primer | Sequence |
|---|---|
| Top (5′–3′) | |
| $T_1$ | GACGCTGCCGAATTCTACCAGTGCCTTGCTAGGACATCTTTGCCCACCTGCAGGTTCACCC |
| $T_2$ | GGAATTCTACCAGTGCCTTGCTAGGACATCTTTGCCCA |
| $T_3$ | GTAGTGCATGTACACCACACTCTTTTTTTTTTTTTT |
| $T_4$ | GACGCTGCCGAATTCTACCAGTGCCTTGCTAGGACATCTTTGCCCA |
| Bottom (3′-5′) | |
| $B_1$ | TGCGACGGCTTAAGATGGTCACGGAACGATGTACCTCGACAGATCTCCTAGGCTGATAGCTA |
| $B_2$ | CTTAAGATGGTCACGGAACGATGTACCTCGACAGATC |
| $B_3$ | CATCACGTACATGTGGTGTGAGTTTTTTTTTTTTTT |
| $B_4$ | CTAGACAGCTCCATGTAGCAAGGCACTGGTAGAATTCGGCAGCGT |

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