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Saccharomyces cerevisiae Hrq1 helicase activity is affected by the sequence but not the length of single-stranded DNA

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ABSTRACT

Mutations in the human RecQ4 DNA helicase are associated with three different diseases characterized by genomic instability. To gain insight into how RecQ4 dysfunction leads to these pathologies, several groups have used the *Saccharomyces cerevisiae* RecQ4 homolog Hrq1 as an experimental model. Hrq1 displays many of the same functions as RecQ4 *in vivo* and *in vitro*. However, there is some disagreement in the literature about the effects of single-stranded DNA (ssDNA) length on Hrq1 helicase activity and the ability of Hrq1 to anneal complementary ssDNA oligonucleotides into duplex DNA. Here, we present a side-by-side comparison of Hrq1 and RecQ4 helicase activity, demonstrating that in both cases, long random-sequence 3′ ssDNA tails inhibit DNA unwinding *in vitro* in a length-dependent manner. This appears to be due to the formation of secondary structures in the random-sequence ssDNA because Hrq1 preferentially unwound poly(dT)-tailed forks independent of ssDNA length. Further, RecQ4 is capable of ssDNA strand annealing and annealing-dependent strand exchange, but Hrq1 lacks these activities. These results establish the importance of DNA sequence in Hrq1 helicase activity, and the absence of Hrq1 strand annealing activity explains the previously identified discrepancies between *S. cerevisiae* Hrq1 and human RecQ4.

1. Introduction

The disease-linked RecQ family helicases are evolutionarily conserved from a single RecQ in bacteria to five RecQs in humans (RecQ1, BLM, WRN, RecQ4, and RecQ5) [1]. Mutations in three RecQs (BLM, WRN, and RecQ4) are directly implicated in human diseases characterized by genomic instability. Unlike BLM and WRN, it is currently unclear how RecQ4 contributes to maintaining genome stability. This is largely due to technical challenges of working with RecQ4 both *in vivo* and *in vitro*. For instance, the presence of a Sld2-like domain (an essential replication initiation factor in yeast) at the *N*-terminus of RecQ4 makes mutational analysis difficult as mutations could disrupt DNA replication [2], which is independent of RecQ4-mediated DNA repair [3]. The Sld2-like domain also confounds biochemical analysis because *Saccharomyces cerevisiae* Sld2 is a known annealase [4], and the Sld2-like portion of RecQ4 plays a significant role in DNA annealing activity [5]. This ability of RecQ4 to anneal complementary DNA substrates is so robust that it initially masked the DNA unwinding activity of the enzyme [6], making RecQ4 helicase activity difficult to analyze. Thus, a model for study of RecQ4 that circumvents these technical challenges was needed.

In-depth bioinformatics revealed *Saccharomyces cerevisiae* Hrq1 as a homolog of RecQ4 [7]. Hrq1 lacks the Sld2-like domain found in metazoan RecQ4 sub-family helicases, alleviating the issue of replication confounding the DNA repair function of the helicase. Furthermore, Hrq1 over-expression and purification produces abundant amounts of protein relative to RecQ4 [8]. Since its discovery, Hrq1 has been determined to play important roles at telomeres such as maintaining telomere length homeostasis in the absence of the known telomerase inhibitor Pif1 [9]. Additionally, Hrq1 functions in the repair of DNA inter-strand crosslinks (ICL) in the same pathway as the ICL repair nuclease Pso2 [8,9]. Telomere maintenance and ICL repair are likewise associated with human RecQ4 [10,11]. *In vitro*, Hrq1 and RecQ4 helicase activity is stimulated by poly(dT) and telomeric repeat ssDNA [8]. Both helicases preferentially bind to Holliday junctions and D-Loop DNA

Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ICL, inter-strand crosslink; EMSA, electrophoretic mobility shift assay.

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structures, with the latter being a preferred substrate for unwinding. Finally, 2D averaging of each helicase via negative staining and transmission electron microscopy shows that both helicases adopt similar tertiary structures, which are distinct from RecQ1.

Concerning DNA unwinding by Hrq1, a weak *in vitro* helicase activity has been reported for the *S. pombe* Hrq1 helicase [12], but the activity displayed by *S. cerevisiae* Hrq1 is more robust [8,9,13]. However, there is a discrepancy in the *S. cerevisiae* Hrq1 literature. Using recombinant Hrq1 over-expressed and purified in two different ways, it has been found that Hrq1 can unwind model fork substrates with ssDNA tails of 25 nt in length. In contrast, another group reports an "absolute requirement for a long 3'-tail (≥70 nt) for efficient unwinding" by Hrq1 [13]. Similarly, it is unclear if Hrq1 lacks single-stranded DNA (ssDNA) annealing activity [8,9] or if it is an annealase like human RecQ4 [13]. The current dearth of Hrq1 literature amplifies these discrepancies and complicates follow-up research as proper substrate design is pivotal for accurately reconstituting biochemical pathways *in vitro*.

Here, we compared the effects of ssDNA tail length and sequence on Hrq1 helicase activity, as well as assayed for annealing activity. We found that increasing the length of the random-sequence ssDNA tails on fork substrates inhibited Hrq1 binding and unwinding, likely due to the higher propensity for longer stretches of ssDNA to form secondary structures. Using equivalent forks with poly(dT) ssDNA tails eliminated length-induced inhibition of Hrq1 helicase activity. However, Hrq1 unwinding of forks with poly(dT)-tails of 25-, 50-, and 75-nt in length was indistinguishable, indicating that long (≥70 nt) 3'-tails are not required for robust helicase activity. Finally, we demonstrated that the strong DNA strand annealing and exchange activities previously reported for RecQ4 [5] are absent in Hrq1.

2. Materials and methods

2.1. Nucleotides, oligonucleotides, and other reagents

^{32}P-ATP was purchased from PerkinElmer (Waltham, MA), and unlabeled ATP was from GE Healthcare (Little Chalfont, UK) or DOT Scientific (Burton, MI). The oligonucleotides used in this work were synthesized by IDT (Coralville, IA) and are listed in Supplementary Table 1. All restriction enzymes were from New England Biolabs (Ipswich, MA).

2.2. Protein purification

S. cerevisiae Hrq1 and human RecQ4 were purified as described [8]. Briefly, the proteins were over-expressed in baculovirus-infected insect cells and purified by nickel affinity (Hrq1) or using amylose resin and Strep-Tactin Superflow (IBA) chromatography. Purification was analyzed by SDS-PAGE and Coomassie staining. Peak fractions were pooled and dialyzed against storage buffer (25 mM Na-HEPES [pH 8], 30% glycerol, 300 mM NaOAc [pH 7.6], 25 mM NaCl, 5 mM MgOAc, 1 mM DTT, and 0.1% Tween-20). Protein concentrations were quantified on SYPRO orange (Sigma) stained SDS-PAGE gels using BSA dilutions to plot a standard curve. Gel imaging and analysis were performed using a Typhoon 9210 Variable Mode Imager (Amersham Biosciences).

2.3. DNA substrates

Substrates were 5'-end labelling with T4 polynucleotide kinase (T4 PNK; NEB) and γ-[^{32}P]-ATP. Labelled oligonucleotides were separated from free label using illustra ProbeQuant G-50 micro columns (GE Healthcare) following the manufacturer's instructions. Oligonucleotides were annealed by incubating complementary or partially complementary oligonucleotides overnight at 37 °C in Annealing Buffer (20 mM Tris-HCl [pH 8], 4% glycerol, 0.1 mM EDTA, 40 μg/mL BSA, 10 mM DTT, and 10 mM MgOAc) [4]. The sequences of the oligonucleotides used to make DNA substrates are listed in Supplementary Table 1.

2.4. DNA binding

Electrophoretic mobility shift assays (EMSAs) were performed for 30 min at 30 °C in 1× binding buffer (25 mM Na-HEPES [pH 8.0], 5% glycerol, 50 mM NaOAc [pH 7.6], 150 μM NaCl, 7.5 mM MgOAc, and 0.01% Tween-20) with 0.1 nM radiolabeled fork substrate. Protein-DNA complexes were separated on 8% 19:1 acrylamide:bis-acrylamide gels at 10 V/cm in TBE (90 mM Tris-HCl [pH 8.0], 90 mM boric acid, and 2 mM EDTA [pH 8.0]). Gels were dried under vacuum and imaged using a Typhoon 9210 Variable Mode Imager. DNA binding was quantified using ImageQuant 5.2 software.

2.5. Helicase assay

DNA unwinding was assessed by incubating the indicated concentrations of helicase with 5 mM ATP, 0.1 nM radiolabelled fork, and 1× unwinding buffer (25 mM Na-HEPES [pH 8.0], 5% glycerol, 50 mM NaOAc [pH 7.6], 150 μM NaCl, 7.5 mM MgOAc, and 0.01% Tween-20). RecQ4 helicase assays were performed in the presence of 15 nM cold ssDNA trap [14]. ATP was required to observe unwinding, suggesting that strand exchange was not the activity being measured (data not shown) [5]. Reactions were incubated at 37 °C for 30 min and stopped with the addition of 1× Stop-Load dye (5% glycerol, 20 mM EDTA, 0.05% SDS, and 0.25% bromophenol blue) supplemented with 400 μg/mL SDS-Proteinase K followed by a 1-min incubation at 37 °C. Unwound DNA was separated on 8% 19:1 acrylamide:bis-acrylamide gels in TBE buffer at 10 V/cm and imaged as for the DNA binding assay.

2.6. DNA annealing and strand exchange assays

Annealing reactions were performed using 0.1 nM each of the partially complementary oligonucleotides MB1057 and MB1059 or MB1167 and MB1168 (Supplementary Table 1) that can be annealed to form a fork. These oligonucleotides were incubated with no protein, Hrq1, or RecQ4 for the indicated times at 37 °C in 1× unwinding buffer. Reactions were stopped by the addition of 1× Stop-Load dye supplemented with 400 μg/mL SDS-Proteinase K and incubated at 37 °C for 1 min. Reactions were then loaded onto 10% 19:1 acrylamide:bis-acrylamide gels, run at 10 V/cm in TBE buffer, and imaged as above.

Strand annealing-dependent strand exchange assays used 0.1 nM fork (the annealed product of oligonucleotides MB1057 and MB1059) incubated with 0.1 nM of oligonucleotide MB1058, which is fully complementary to MB1057 such that strand exchange results in conversion of the forked structure into a faster-migrating blunt ended double-stranded DNA (dsDNA) substrate. These assays were performed in the same conditions as for annealing activity, and products were separated on 12% 19:1 acrylamide:bis-acrylamide gels in TBE buffer at 10 V/cm and imaged as above. The fork oligonucleotides used in the strand annealing and strand exchange assays were identical to those used previously to identify RecQ4-mediated strand exchange activity [5].

2.7. Statistical analyses

All data were analyzed and plotted using GraphPad Prism 6 (GraphPad Software, Inc). The plotted values are averages, and the error bars were calculated as the standard deviation from three or