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Saccharomyces cerevisiae Hrq1 requires a long 3′-tailed DNA substrate for helicase activity

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ABSTRACT

RecQ helicases are well conserved proteins from bacteria to human and function in various DNA metabolism for maintenance of genome stability. Five RecQ helicases are found in humans, whereas only one RecQ helicase has been described in lower eukaryotes. However, recent studies predicted the presence of a second RecQ helicase, Hrq1, in fungal genomes and verified it as a functional gene in fission yeast. Here we show that 3′–5′ helicase activity is intrinsically associated with Hrq1 of *Saccharomyces cerevisiae*. We also determined several biochemical properties of Hrq1 helicase distinguishable from those of other RecQ helicase members. Hrq1 is able to unwind relatively long duplex DNA up to 120-bp and is significantly stimulated by a preexisting fork structure. Further, the most striking feature of Hrq1 is its absolute requirement for a long 3′-tail ($\geqslant$70-nt) for efficient unwinding of duplex DNA. We also found that Hrq1 has potent DNA strand annealing activity. Our results indicate that Hrq1 has vigorous helicase activity that deserves further characterization to expand our understanding of RecQ helicases.

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1. Introduction

RecQ helicases are a family of conserved enzymes found in all living organisms that play multiple roles in the maintaining genome stability. They all contain a highly conserved helicase domain and unwind DNA in the 3′–5′ direction [1,2]. While bacteria possess only a single RecQ helicase, mammals, including humans, have five RecQ family members, designated RECQL1, WRN, BLM, RECQL4, and RECQL5. Mutations in three of these genes (BLM, WRN, and RECQL4) are responsible for heritable diseases, Bloom syndrome, Werner syndrome, and Rothmund–Thomson syndrome (RTS), respectively [3]. They are rare recessive Mendelian diseases that share common clinical features, including premature aging, cancer predisposition, and genome instability [1,4].

BLM and its yeast orthologue Sgs1 are versatile helicases that function at various steps in homologous recombination (HR) repair pathways, such as dissolution of double Holliday junction and 5′-end resection [2,3,5]. They also play important roles in the stabilization of polymerases at stalled replication forks and HR-mediated replication restart [6,7]. WRN also has been extensively characterized and known to play multiple functions in the recovery of stalled replication forks, DNA repair, transcription, and telomere maintenance [8,9]. Compared to BLM and WRN, RECQL4 has been poorly characterized. Human RECQL4 was only recently proven to possess helicase activity *in vitro* [10–12]. RECQL4 appears to be involved in both repair pathways and the initiation of DNA replication. Cells from some RTS patients were sensitive to UV and ionizing radiation, and supported DNA repair synthesis poorly, suggesting that RECQL4 is involved in several DNA repair pathways [13]. However, the biological functions of RECQL4 that maintain genome integrity remain to be elucidated.

Until recently, only one RecQ orthologue was described in lower eukaryotes and plants [3,5]. Sgs1 and Rqh1, similar to human BLM, were considered as the only RecQ family member in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively. Although *S. pombe* contains other RecQ-like proteins (Tlh1 and Tlh2), they appear to participate in telomere metabolism only during crisis of telomere erosion [14]. However, bioinformatics analyses have predicted the presence of another highly conserved RecQ-homologous protein, Hrq1 (*H*omologous to *RecQ* helicase *1*), in the fungal and plant genomes, which is most similar to metazoan RECQL4 [15]. This prediction has been supported by recent studies in *S. pombe* that showed that Hrq1-deficient mutant cells suffer spontaneous genome instability and the purified Hrq1 protein contained 3′–5′ helicase activity [16].

In this study, we purified and characterized the recombinant Hrq1 protein of *S. cerevisiae* expressed in insect cells using the baculovirus system. In this report, we demonstrate that the purified Hrq1 protein possesses a moderately processive helicase activity that is stimulated by a long 3′-tail length and a fork structure. We also show that Hrq1 has DNA strand annealing activity.

Abbreviations: RTS, Rothmund–Thomson syndrome; HR, homologous recombination; sscDNA, single-stranded circular DNA; SSB, ssDNA-binding protein.

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2. Materials and methods

2.1. Preparation of oligonucleotides and DNA substrates

All oligonucleotides utilized for construction of DNA substrates were commercially synthesized (Bioneer, Korea) and were gel-purified prior to use. The sequence of oligonucleotides used in this study are listed in Supplementary Table S1. The 5′-ends of oligonucleotides were labeled by the incorporation of [γ-^{32}P] ATP using T4 polynucleotide kinase, and the substrates were prepared as described [17]. The ^{32}P-labeled oligonucleotides are denoted by asterisks below. For the construction of the ΦX174-based partial duplex (240-bp) substrates, a 240-bp fragment was amplified by PCR in the presence of ^{32}P-labeled primer H7 and unlabeled primer H10 using ΦX174 RF DNA as a template. The fragment was purified and annealed to ΦX174 single-stranded circular (ssc) DNA, followed by purification as described [17].

2.2. Cloning and purification of Hrq1

The open reading frame of *HRQ1* gene from *S. cerevisiae* was amplified by PCR and cloned into the *Sac*I–*Not*I sites of pFastBacHTc plasmid (Invitrogen, Carlsbad, CA) with an N-terminal Strep-tag (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys). The Hrq1K318A mutant was generated by site-directed mutagenesis using the Quick Change system (Stratagene, La Jolla, CA).

A recombinant baculovirus was constructed in order to produce Hrq1 protein as recommended by the manufacturer (Invitrogen, Carlsbad, CA). Sf9 insect cells (1×10^6 cells/ml) were infected at a multiplicity of infection of 1 and grown for an additional 70 h. Cells were then harvested, resuspended in 50 ml of buffer T (25 mM Tris–HCl, pH 7.5, 10% glycerol, 0.1 mM PMSF, and 0.15 μg/ml each of leupeptin and pepstatin A) containing 300 mM NaCl, and disrupted by sonication (5 cycles of a 30 s pulse and 1 min cooling interval). The extract was cleared by centrifugation at 45,000 rpm for 1 h, and the supernatant directly applied to a Ni^{2+}-NTA-agarose (Qiagen, Valencia, CA) column (1.77 $cm^2 \times 2.5$ cm, 4.5 ml) equilibrated with buffer T_{300} (hereafter, the number indicates the concentration of NaCl in buffer T) plus 20 mM imidazole. The column was washed consecutively with 5 column volumes of the same buffer, and then eluted with 250 mM imidazole in the same buffer. Peak fractions containing Hrq1 were pooled and incubated with Strep-Tactin-Sepharose resin (0.5 ml, GE Healthcare Life Sciences, Pittsburgh, PA) for 1 h. The resin was then washed 3 times with 50 ml of buffer T_{300}, and the protein eluted 5 times with 0.5 ml of buffer T_{300} containing 10 mM desthiobiotin. The fractions were pooled, concentrated, and then loaded onto a glycerol gradient (5 ml, 15–35% glycerol in buffer T_{500}). The gradient was subjected to centrifugation for 24 h at 48,000 rpm in a Beckman SW55 Ti rotor. Fractions (200 μl) were collected from the bottom of the gradient and assayed for helicase activity.

2.3. Helicase, DNA strand annealing, and DNA-binding assays

Helicase assays were carried out in reaction mixtures (20 μl) containing 50 mM Tris–HCl (pH 7.0), 10 mM $MgCl_2$, 2.5 mM ATP, 0.5 mM DTT, 0.25 mg/ml bovine serum albumin, and 2.5 fmol of DNA substrate. Reactions were initiated by enzyme addition; after 30 min of incubation at 37 °C, reactions were stopped with 4 μl of 6× stop solution (60 mM EDTA, 40% sucrose, 0.6% SDS, 0.25% bromophenol blue, and 0.25% xylene cyanol). The reaction products were subjected to 10% polyacrylamide gel containing 0.1% SDS in 1× TBE. The gels were then dried on DEAE-cellulose paper and subjected to autoradiography. The quantity of labeled DNA products was then determined using a PhosphorImager.

The reaction conditions used to examine DNA strand annealing and DNA-binding activities were similar to those described previously [18]. For DNA strand annealing assays, reaction mixtures containing 2.5 fmol of ^{32}P-labeled single-stranded (ss) DNA and 2.5 fmol of unlabeled complementary ssDNA were incubated at 37 °C for the indicated time, followed by separation on 10% PAGE. For DNA-binding assays, reactions were incubated for 15 min at 37 °C, and the resulting DNA–protein complexes separated on 6% PAGE in 0.5× TBE in the absence of SDS.

3. Results

3.1. Purified Hrq1 protein contains DNA helicase activity

Sequence analyses of Hrq1 [15], as well as the recent study of *S. pombe* Hrq1 [16], suggest that fungal and plant Hrq1 proteins are new members of the RecQ family of helicases. Hrq1 sequences are found in all fungal genomes including *S. cerevisiae*, and the phylogenetic analysis indicates that Hrq1 sequences are most similar to RECQL4 sequences among the RecQ family [15]. In this study, we purified *S. cerevisiae* Hrq1 protein in order to determine whether RECQL4-like helicase activity is intrinsically associated with this protein. For this purpose, a recombinant Hrq1 protein containing N-terminal His- and Strep-tags was expressed in insect Sf9 cells using the baculovirus system. The protein formed was purified using consecutive Ni^{2+} and Strep-Tactin columns, followed by additional purification via 15–35% glycerol gradient centrifugation as described in Section 2 (Figs. 1A and S1). The purified protein exhibited DNA helicase activity that displaced the 20-mer oligonucleotide

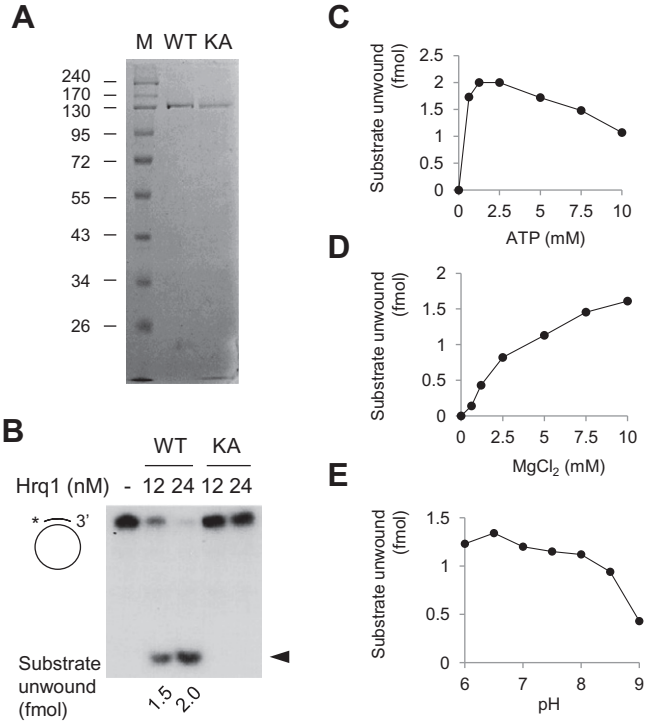

Fig. 1. Purification and helicase activity of Hrq1 protein. (A) Coomassie staining of purified Hrq1 (1 μg, WT) and Hrq1K318A (1 μg, KA) separated on SDS–PAGE (10%). M denotes molecular size markers. (B) Helicase activity of Hrq1. DNA unwinding reactions were performed for 30 min at 37 °C by incubation of the indicated amount of enzyme with 2.5 fmol of oligonucleotide H1 annealed to ΦX174 sscDNA. The products were analyzed on 10% polyacrylamide gel and the resulting autoradiogram is shown. The arrowhead indicates the position where unwound substrate migrated. The amounts of unwound substrate are presented at the bottom of the figure. (C–E) Effects of various concentrations of ATP (C) and Mg^{2+} (D), and different pHs (E), on the helicase activity. Helicase assays were carried out with 24 nM of Hrq1 as in (B), and the results are plotted.

annealed to ΦX174 sscDNA (Fig. 1B). In addition, SDS–PAGE analysis accompanied by helicase and ATPase assays of the glycerol gradient fractions revealed co-purification of Hrq1 protein, with the ssDNA-dependent ATPase and helicase activities (Supplementary Fig. S1). The amount of products formed was proportional to the concentration of Hrq1 in each fraction, indicating that both activities are associated with Hrq1. To confirm that helicase activity is intrinsic to Hrq1, we also produced a mutant protein, Hrq1K318A, that contained a substitution (lysine to alanine substitution at amino acid 318) in the ATP-binding motif (Walker A motif) and purified exactly as the wild-type Hrq1 (Fig. 1A). While the wild-type Hrq1 protein efficiently unwound the substrate, Hrq1K318A was completely devoid of helicase (Fig. 1B) and ATPase activities (data not shown), demonstrating that the both activities reside in Hrq1.

To optimize the unwinding reaction catalyzed by Hrq1, the concentrations of ATP and Mg^{2+} were titrated in the reaction. The helicase activity of Hrq1 was measured at various concentrations of ATP and Mg^{2+} with the same substrate used in Fig. 1B. The activity was optimal in the presence of 1–2.5 mM ATP and decreased at higher ATP levels (Fig. 1C). In contrast, the activity increased in proportion to the concentration of Mg^{2+} (Fig. 1D). We also examined the pH dependency of helicase activity. High levels of activity were detected with the pH range of 6–8 but rapidly decreased at higher pH (Fig. 1E). Based on these results, all subsequent unwinding reactions were carried out at pH 7.0 with 2.5 mM ATP and 10 mM $MgCl_2$.

3.2. Hrq1 is a 3′–5′ helicase and its activity is stimulated by fork structures

We next determined the polarity of Hrq1 helicase activity using 5′- and 3′-overhang substrates. As all members of the RecQ family are 3′–5′ helicases [2,3], Hrq1 unwound the 3′-overhang only, indicating that it translocates in the 3′–5′ direction (Fig. 2A). Recently, it was shown that *S. pombe* Hrq1 also exhibits 3′–5′ polarity [16].

To examine the influence of a preexisting fork structure on the unwinding activity, we compared the unwinding efficiency of a fork-structured substrate containing a 5′-tail (25-nt) with that of a flush-structured substrate. Theoretically, Hrq1 can interact with this fork structure when it translocates in the 3′–5′ direction along the template DNA. A fork structure was found to significantly stimulate (about 2-fold) the helicase activity of Hrq1 (Fig. 2B). Consistent with this observation, Y-shaped substrates constructed from two oligonucleotides were unwound by Hrq1 more efficiently than the 3′-overhang substrate (data not shown). We next examined the effect of ssDNA-binding protein (SSB) on the helicase activity. In contrast to BLM, WRN, and Sgs1 that are stimulated by RPA (eukaryotic SSB) [19–21], helicase activity of Hrq1 was significantly inhibited by the addition of RPA (Fig. 2B), implying that the RPA-coated ssDNA is inaccessible to Hrq1 and there is no specific interaction between RPA and Hrq1. To investigate substrate preference of Hrq1, helicase activity was tested using various DNA structures shown in Fig. 2C. We found that only fork-structured substrate was unwound, indicating that Hrq1 cannot utilize branched DNA structures except simple Y-shaped DNA.

3.3. Hrq1 requires a long 3′-tail length for helicase activity

Interestingly, unwinding reactions with ΦX174-based substrates were more efficient than those formed with oligonucleotide substrates (compare Fig. 2A and B). Hrq1 exhibited only marginal activity with oligonucleotide substrates, even in the presence of excess levels of Hrq1 protein (50 nM, 400-fold excess over

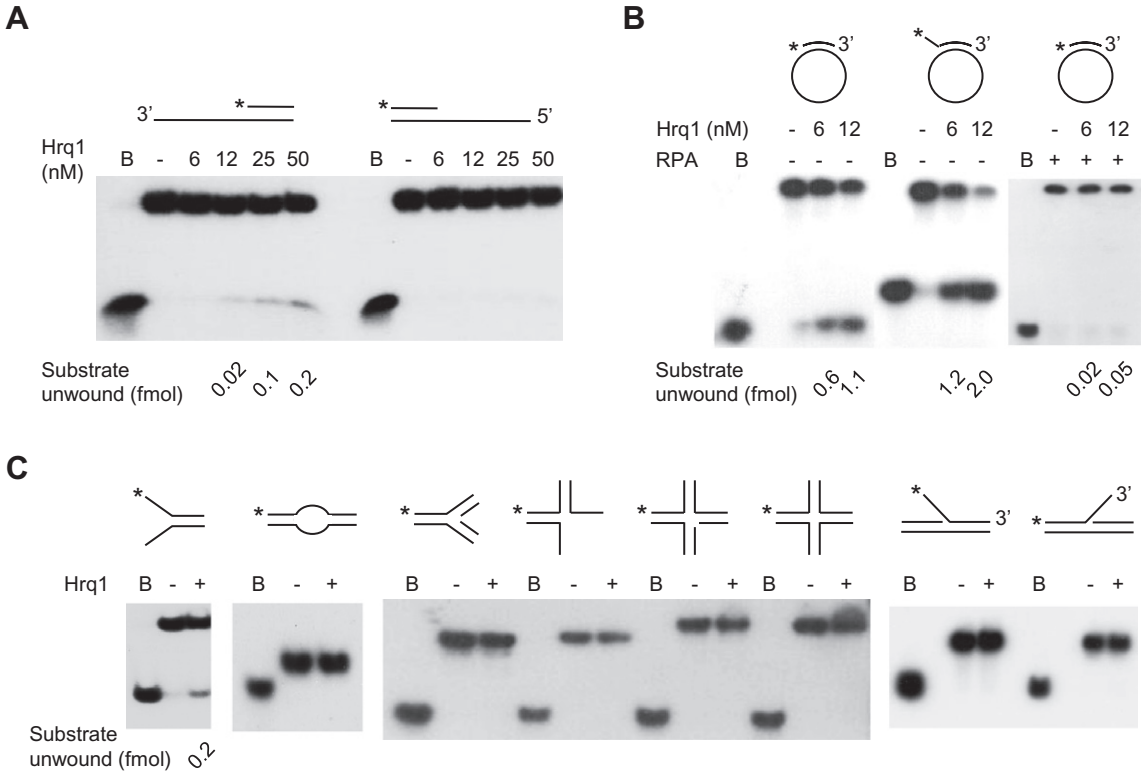

Fig. 2. Properties of Hrq1 helicase activity. (A) Polarity of DNA unwinding. The ^{32}P-labeled H1 and H2 oligonucleotides were annealed to H5 to form 3′- and 5′-overhang substrates, respectively. Helicase assays were performed with 2.5 fmol of the substrates shown at the top of the figure and the indicated levels of Hrq1. The products were analyzed on 10% PAGE and the resulting autoradiogram is shown. B denotes boiled substrate controls. The amount of product formed is presented at the bottom of the figure. (B) Preference for a fork-structured substrate. Oligonucleotides H1 and H3 were annealed to ΦX174 sscDNA to generate a substrate with a flush end (left and right) or a 5′-tail (25-nt, middle), respectively. Helicase reactions were carried out in the presence (+) and absence (−) of RPA (240 ng, 100 nM). (C) Helicase activity on various DNA substrates. Constructions of the DNA substrates are described in Supplementary Table S1. Helicase reactions were carried out with 50 nM of Hrq1.

substrate; Fig. 2A). In contrast, ΦX174-based substrates were unwound efficiently in the presence of much lower levels of Hrq1 protein (12 nM; Fig. 2B). One striking difference between these two types of substrates is the large amount of ssDNA (~5200-nt) present in ΦX174 substrate. Another notable difference is the free 3′-ssDNA end of oligonucleotide substrates, which is not present on the ΦX174 sscDNA.

To distinguish which structural difference affected the Hrq1 helicase activity, 3′-overhang substrates with identical duplex regions (20-bp) but containing varying 3′-tail lengths were prepared, as shown in Fig. 3. Using these substrates, we found that the unwinding activity of Hrq1 was significantly stimulated as the length of ssDNA increased (Fig. 3A). The substrate containing a 30-nt tail was hardly unwound by Hrq1 (lanes 2–4). However a substantial amount of the substrate was unwound by the presence of a 70-nt tail (lanes 6–8), and the unwinding was further increased using a tail of 180-nt (lanes 10–12). We also compared the binding of Hrq1 to these substrates by gel mobility shift assays. When the 30-nt tailed substrate was tested, DNA–protein complexes were not detectable (Fig. 3B, lanes 2–4). In contrast, stable complexes were detected with the 70-nt tailed substrate at the highest level of Hrq1 (lane 8). Complex formation became more efficient with the 180-nt tailed substrate (lanes 10–12). These results suggest that both substrate-binding and helicase activities of Hrq1 are elevated as the length of the 3′-tail increases. To examine the effect of the 3′-ssDNA end, we prepared a substrate identical to the 3′-overhang with 70-nt tail (Fig. 3) but containing duplexes (20-bp) at both ends. The unwinding efficiency of this substrate was almost the same as that of the 3′-overhang (data not shown). This result indicates that a free ssDNA end does not affect the helicase activity. However, we cannot exclude the possibility that an ssDNA end is inhibitory to the enzyme activities because the ΦX174 substrate was utilized still more efficiently than the oligonucleotide substrate containing a 180-nt tail (compare Figs. 2B and 3A).

It is noteworthy that Hrq1 did not bind to the blunt-ended dsDNA (50-bp) though it formed stable DNA–protein complexes with ssDNA of the same length (data not shown). This observation suggests that Hrq1 requires ssDNA on which it is loaded before acting as helicase. If Hrq1 has no sequence preference for ssDNA-binding, a substrate with longer 3′-tail will provide more ssDNA for Hrq1 recruitment and the unwinding reaction.

3.4. Hrq1 has both processive helicase and DNA strand annealing activities

As shown in Fig. 1B, Hrq1 nearly completely unwound short duplex DNA (20-bp) under our helicase assay condition. This vigorous helicase activity encouraged us to examine the unwinding of longer duplex DNA. We found that Hrq1 unwound duplex DNA as long as 120-bp (Fig. 4A). However, the unwinding reaction was significantly compromised as the duplex DNA length was increased, and the longest duplex DNA examined (240-bp) was not displaced. Nevertheless, the processivity of Hrq1 exceeded our expectation as human RECQL4 possesses poor processive helicase activity that can displace only short oligonucleotides (⩽22-nt) [11,12].

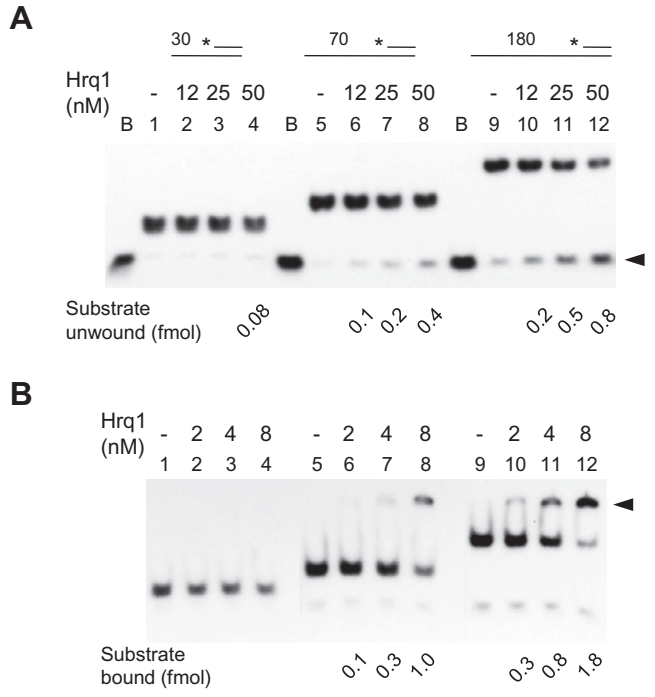

Fig. 3. Stimulation of helicase activity by a long 3′-tail. (A) Effect of 3′-tail length on DNA unwinding activity. The ^{32}P-labeled H1 was annealed to H4, H5, and H6 to construct partial duplex DNAs with different 3′-tail lengths (30-, 70-, and 180-nt, respectively). Helicase reactions were carried out with 2.5 fmol of each substrate shown at the top of the figure. B denotes boiled substrate controls. The arrowhead indicates the position where the unwound substrate migrated. The amounts of products are indicated at the bottom of the figure. (B) Length-dependent substrate binding of Hrq1. For gel mobility shift assays, indicated amounts of Hrq1 were incubated with 2.5 fmol of the same substrates as in (A), and the resulting DNA–protein complexes separated on 6% non-denaturing PAGE. The arrowhead indicates the migration positions of the DNA–Hrq1 complexes. The amounts of products are indicated at the bottom of the figure.

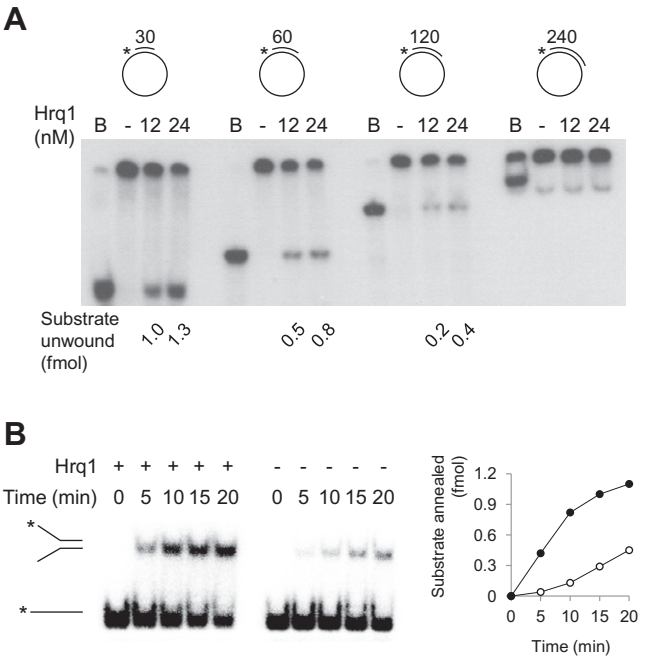

Fig. 4. Hrq1 possesses both processive helicase and DNA strand annealing activities. (A) Processivity of helicase activity. Oligonucleotides H7, H8, H9, and a PCR-amplified 240-nt ssDNA were annealed to ΦX174 sscDNA to construct the 30-, 60-, 120-, and 240-bp partial duplex substrates, respectively. Reactions were carried out with the indicated amounts of Hrq1, and the amounts of products are indicated at the bottom of the figure. B denotes boiled substrate controls. (B) DNA strand annealing activity of Hrq1. DNA strand annealing assays were performed in the presence (+) and absence (−) of Hrq1 (15 ng, 6 nM) with 2.5 fmol of ^{32}P-labeled H3 and unlabeled H4 for the indicated times at 37 °C. The products were analyzed on 10% PAGE, and the resulting autoradiogram is shown at left. Schematic structure of the expected product is indicated at the migration positions of the products. The amounts of substrate annealed in the presence (●) and absence (○) of Hrq1 were measured and plotted at right.

The low processivity of RECQL4 is partly due to its strong DNA strand annealing activity, which counteracts its helicase activity [10,22]. Therefore, we investigated if Hrq1 also exhibited DNA strand annealing activity. Fig. 4B shows that the annealing of complementary oligonucleotides by Hrq1 was significant. The annealing reaction was ATP-independent and approximately 3 times faster than the spontaneous annealing of the complementary DNA (Fig. 4B). In addition, the rate of the annealing reaction was proportional to the concentration of Hrq1 added (data not shown), indicating that the annealing activity is intrinsic to Hrq1. Although many RecQ helicase preparations have DNA strand annealing activity, its biological function remains unclear.

4. Discussion

In this study, we show that Hrq1 of *S. cerevisiae* has intrinsic helicase. This activity co-purified with the recombinant Hrq1 protein and a mutation in the conserved ATP-binding motif abolished the helicase activity. We also demonstrated a number of biochemical properties of Hrq1, including its DNA-binding and DNA strand annealing activities. A unique biochemical property of Hrq1 noted in this study is the 3′-tail length stimulation of helicase activity. The stimulation was roughly proportional to the length of ssDNA, which most likely increased the recruitment of Hrq1. This is a curious property for a helicase, in that it is unlikely that naked ssDNA regions long enough to support helicase activity of Hrq1 exist *in vivo*. Instead, Hrq1 may require specific DNA structures for targeting, considering that certain RecQ helicases preferentially unwind specific DNA structures. For example, Sgs1 utilizes a broad range of DNA substrates but prefers a Holliday junction [21]. As shown in Fig. 2C, however, no substrates without long 3′-tails were unwound, indicating that Hrq1 is absolutely dependent on the length of 3′-tail. Nevertheless, we cannot exclude the possibility that Hrq1 has a preference for certain types of DNA structures or sequences. Human RecQ helicases BLM (and its yeast orthologue Sgs1) and WRN are capable of resolving telomeric secondary structures such as G-quadruplexes and telomeric D-loops [1,2,21]. A recent study showed that RECQL4 can displace telomeric D-loops, implicating a role for RECQL4 in telomere maintenance [23]. These observations hint that Hrq1 may also utilize yeast telomeric DNA and/or interact with telomeric DNA-binding proteins.

Human RecQ helicases, except for RECQL4, exhibit moderate processivity that unwinds medium duplex DNA length ($\leqslant$100-bp), but they can displace longer duplexes with the assistance of SSB. For example, RPA enables the full-length Sgs1 to unwind dsDNA as long as 23-kb [21]. The stimulation of helicase activity by SSB is likely due to trapping ssDNA and preventing it from reannealing because *Escherichia coli* SSB and yeast RPA have similar stimulatory effects [21]. However, BLM and WRN are stimulated by a specific interaction with human RPA [19,20]. Accordingly, we predicted that, similar to other RecQ helicases, Hrq1 might be stimulated by RPA. However, we found that yeast RPA inhibited the Hrq1 helicase activity using ΦX174-based substrate (Fig. 2B).

Recently it was reported that RECQL4 interacts with both BLM and WRN *in vivo* and *in vitro*, and stimulates their helicase activities on fork substrates [24] and on telomeric D-loops [23]. These physical and functional interactions raise the possibility that Hrq1 may interact with Sgs1 in a similar manner. Another candidate for Hrq1-interacting protein is Sld2, which plays an essential role in the initiation of DNA replication in *S. cerevisiae* [13,25,26]. The N-terminal region of RECLQ4 is homologous to yeast Sld2, but Hrq1 lacks this Sld2-like domain. It is interesting that the N-terminal and the other helicase domain of RECQL4 are homologous to two separate proteins, Sld2 and Hrq1, respectively. Thus, we speculate that Hrq1 may interact with Sld2 and contribute to the initiation of DNA replication.

Although human RECQL4 has been studied extensively, little is known about the helicase properties of this enzyme. This is partly due to the poor helicase activity that makes it difficult to characterize its properties. In this study, we show that Hrq1 has advantages over RECQL4 for biochemical characterization of helicase activity. Therefore, further studies of Hrq1 will improve our knowledge of the enzyme properties and *in vivo* function of human RECQL4.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.109.

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