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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.

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

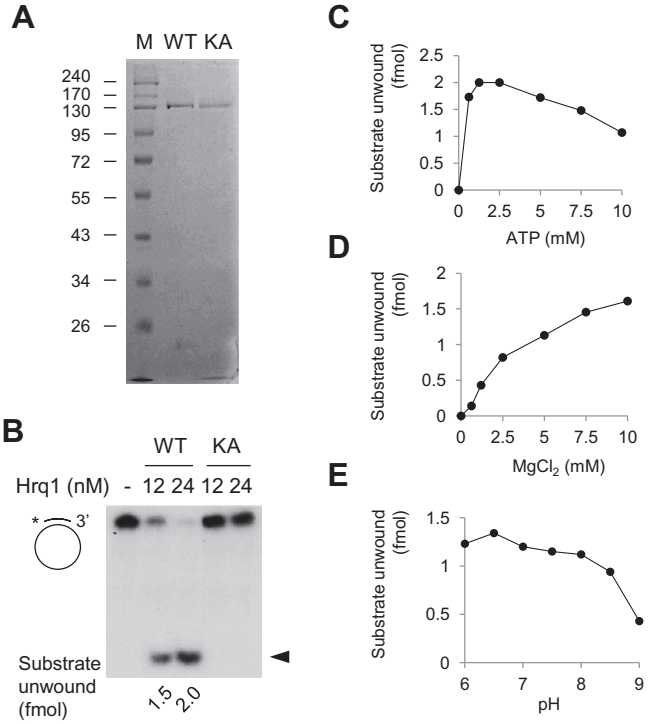

Fig. 1. Purification and helicase activity of Hrq1 protein. (A) Coomassie staining of purified Hrq1 (1 μg, WT) and Hrq1K318A (1 μg, KA) separated on SDS–PAGE (10%). M denotes molecular size markers. (B) Helicase activity of Hrq1. DNA unwinding reactions were performed for 30 min at 37 °C by incubation 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.