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DNA Helicase Activity in Purified Human RECQL4 Protein

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Human RECQL4 protein was expressed in insect cells using a baculovirus protein expression system and it was purified to near homogeneity. The protein sedimented at a position between catalase (230 kDa) and ferritin (440 kDa) in glycerol gradient centrifugation, suggesting that it forms homo-multimers. Activity to displace annealed 17-mer oligonucleotide in the presence of ATP was co-sedimented with hRECQL4 protein. In ion-exchange chromatography, both DNA helicase activity and single-stranded DNA-dependent ATPase activity were co-eluted with hRECQL4 protein. The requirements of ATP and Mg for the helicase activity were different from those for the ATPase activity. The data suggest that the helicase migrates on single-stranded DNA in a 3′–5′ direction. These results suggest that the hRECQL4 protein exhibits DNA helicase activity.

Key words: DNA helicase, DNA replication, RecQ helicase, ATPase, Rothmund–Thomson syndrome.

Abbreviations: WRN, Werner's syndrome; MCM, minichromosome maintenance; Sld2, synthetic lethality with dpbll-12.

RecQ-family proteins that have conserved RecQ-type DNA helicase domains play a role in maintaining genome integrity, and five human RECQL family members (RECQL1-5) have been identified (*1–3*). Genetic dysfunction in human WRN protein (RECQL2 or RECQ3) and Bloom protein (RECQL3 or RECQ2) results in premature aging and cancer-prone syndromes; furthermore, genome instability is associated with these syndromes. These two proteins exhibit DNA helicase activity *in vitro*. Human RECQL1 (*4*, *5*) and QL5 (*6*) proteins also exhibit DNA helicase activity *in vitro*, suggesting that they are all involved in the regulation of DNA recombination by catalyzing the separation or annealing of DNA strands *in vivo*. Mutations in human *RECQL4* genes can lead to Rothmund–Thomson syndrome (*7*) which is associated with a cancer-prone phenotype with highly frequent genetic disorders, suggesting that the protein is also involved in maintaining genomic stability (*8*). It remains to be addressed whether human RECQL4 protein exhibits DNA helicase activity *in vitro*.

In addition to the critical roles in the regulation of genomic recombination, it has been recently shown that *Xenopus* RecQL4 (XRecQL4) protein is required for the initiation of DNA replication in a DNA-replication system using egg extracts (*9*). The data suggest that XRecQL4 protein is involved in the DNA unwinding of the DNA duplex in the initiation of DNA replication, since the protein is required for the assembly of RPA, a single-stranded DNA-binding protein, to DNA. In contrast, XRecQL4 protein is not required for the assembly of MCM2-7 complex, which is postulated to be replicative DNA helicase, to DNA. It was found that a mutant XRecQL4 protein where the ATP-binding motif is mutated is not able to support the DNA replication in this system; this suggests that XRecQL4 protein functions as a DNA helicase in the initiation of DNA replication. In addition to the DNA helicase domains, XRecQL4 protein has a region homologous to Sld2 protein in the amino-terminal portion (*9*, *10*). Sld2 protein in *Saccharomyces cerevisiae* is required for the assembly of the GINS complex in the initiation region (*11*). GINS, which is a complex of Sld5, Pfs1, Psf2 and Psf3 proteins, interacts with MCM2-7 complex and Cdc45 protein; the resultant CMG complex appears to function as a replicative DNA helicase *in vivo* (*12*). Thus, XRecQL4 protein may have a function in assembling the GINS complex in the initiation region, in addition to serving the function as a DNA unwinding enzyme.

In the *Xenopus* DNA-replication system, however, it has been reported that the amino-terminal fragments of XRecQL4 protein can compensate for the defect in DNA-replication activity of XRecQL4-depleted egg extracts (*10*); this suggests that the region homologous to Sld2 protein, but not DNA helicase domains, plays an essential role in the DNA replication. However, it appears that the complementation by the XRecQL4 fragments is not complete in supporting the DNA replication, since the DNA-replication activity was not fully recovered, even in the presence of excess amounts of the fragments. Thus, it remains to be determined, whether the presumptive DNA helicase activity of XRecQL4 protein is required for the initiation of DNA replication.

In this study, we expressed human RECQL4 (hRECQL4) protein in a baculovirus protein expression system and purified it to near homogeneity. The hRECQL4 protein has a region homologous to Sld2

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protein in the amino-terminal region, as well as in the conserved helicase domains. The data indicate that both the DNA helicase activity and a single-stranded DNA-dependent ATPase activity are associated with the hRECQL4 protein. The requirements of ATP and Mg for the helicase activity were different from those for the ATPase activity. The helicase appears to migrate in a 3′–5′ direction on single-stranded DNA. These results are discussed in relation to other works.

MATERIALS AND METHODS

Cloning of Human RECQL4 Gene and its Expression—Human *RECQL4* cDNA was synthesized from mRNA of HeLa cells, and the gene was amplified via the RT–PCR method (Invitrogen, Carlsbad, CA, USA) in the presence of 1 M betain (*13*). The *hRECQL4* gene was cloned into pcDNA3.1 vector (Invitrogen) between *Eco*RV and *Not*I sites. Proteins were expressed from the cloned genes by a transcription-coupled translation system using rabbit reticulocyte lysate (Promega, Madison, WI, USA). About 150 kDa of protein was expressed from one clone and the clone was re-cloned in a baculovirus vector (pVL1393) between *Sma*I and *Not*I sites to be expressed as a flag-hRECQL4 fusion protein where a flag peptide (DYKDDDDK) is attached to the amino-terminus of the hRECQL4 protein. The nucleotide sequence of the cloned hRECQL4 gene was determined by DNA sequencing in OpenGene system (Veritas, Tokyo, Japan). A recombinant baculovirus was prepared according to the manufacturer's protocol (Pharmingen, BD, San Jose, CA, USA). For expression of the protein, Sf9 cells on ϕ150-mm dishes were infected with the virus for 2 days.

The recombinant hRECQL4 protein in infected cell lysate was firstly purified using anti-flag antibody agarose (Sigma, St Louis, MO, USA), as follows. The infected cells were suspended in lysis buffer consisting of 10 mM Tris–HCl (pH 7.5), 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM Na phosphate buffer, 10 mM $Na_4P_2O_7$ and protease inhibitors (Pharmingen, BD, San Jose, CA, USA). After incubation for 40 min on ice, insoluble material was removed by centrifugation at 40,000 rpm (TLS55, Beckman, Fullerton, CA, USA) for 40 min at 4°C. To 2 ml of the clarified lysate, 0.4 ml of anti-flag antibody agarose was added, and the mixture was incubated for 3 h at 4°C on a rocking platform. The beads were then collected by centrifugation and washed three times with TBS [50 mM Tris–HCl (pH 7.5) and 150 mM NaCl]). The agarose was incubated with an equal volume of TBS containing 50 μg/ml flag peptide (Sigma). This was followed by incubation for 15 min at 4°C on a rocking platform and the removal of the beads via centrifugation. The elution of proteins was repeated two more times. The pooled eluates were concentrated with a Microcon 30 (Millipore, Bedford, MA, USA) and the proteins were separated by centrifuging at 36,000 rpm for 14 h in 15–30% glycerol gradient containing 20 mM Tris–HCl, 0.5 mM EDTA, 150 mM NaCl and 0.01% Triton X-100. After fractionation, proteins in the fractions were analysed by SDS–polyacrylamide gel (8%) electrophoresis and they were stained with silver.

As another method, the proteins purified using the anti-flag antibody agarose were loaded onto a MonoQ or MonoS column in a Smart system (GE Healthcare, Piscataway, NJ, USA), and the bound proteins were eluted by a linear gradient from 0.1 M to 0.6 M NaCl. Proteins in eluted fractions were analysed by SDS–polyacrylamide gel electrophoresis and they were stained with 0.1% coomassie brilliant blue in 10% acetic acid and 40% methyl alcohol. The gel was destained in 7% acetic acid and 5% methyl alcohol.

Immuno-Blotting—Proteins containing hRECQL4 protein were separated by SDS–polyacrylamide gel (8%) electrophoresis. After the proteins in the gel were transferred to Immobilon-P transfer membrane (Millipore), the membrane was incubated for 1 h at room temperature with a blocking buffer (Ez Block, ATTO, Tokyo, Japan) diluted by 3-fold with TBS plus 0.1% Triton X-100; it was then incubated overnight at 4°C with 1 μg/ml of anti-flag mouse antibody (Sigma) in the diluted blocking buffer. After washing the membrane with TBS containing Triton X-100, it was incubated for 2 h at 27°C with anti-mouse antibody conjugated with horse radish peroxidase (BioRad, Hercules, CA, USA). After washing, the membrane was incubated with SuperSignal West Pico Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) and a chemiluminescent signal was detected by Light-Capture (ATTO).

DNA Helicase and ATPase Activities in Purified hRECQL4 Protein—The DNA helicase substrates were prepared as reported (*14*), except that 5′-labelled oligonucleotides annealed to M13 mp18 single-stranded DNA were purified by centrifuging five times through a spin column (G-50, Roche, Mannheim, Germany), instead of being purified via sucrose gradient centrifugation. A 17 mer (5′-GTTTTCCCAGTCACGAC-3′), a 37 mer (5′-TCG ACTCTAGAGGATCCCCGGGTACCGAGCTCGAATT-3′) and a 53 mer (5′-CCAAGCTTGCATGCCTGCAGGTCGAC TCTAGAGGATCCCCGGGTACCGAGCTC-3′) oligonucleotide were used and their 5′ ends were labelled with polynucleotide kinase in the presence of [γ-^{32}P]ATP, before annealing to mp18 DNA. DNA helicase activity was measured essentially as reported previously (*14*). The reaction contained 10 mM ATP, 10 mM $Mg(OAc)_2$ and 1∼2.5 fmol of 17-mer oligonucleotide annealed to M13 mp18 DNA. For measuring ATPase activity, hRECQL4 protein was incubated at 37°C for 30 min with 2 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) in the reactions containing 50 mM Tris–HCl (pH 7.9), 20 mM β-mercaptoethanol, 0.5 mg/ml bovine serum albumin, 5 μg of single-stranded DNA (heat-denatured), 10 mM $Mg(OAc)_2$ and 10 mM ATP. Then, 0.5 μl of the reaction was spotted on a poly(ethyleneimine)-cellulose thin layer chromatography plate (Cellulose F, Merck, Darmstadt, Germany). The chromatography was carried out at 4°C in 0.8 M acetic acid and 0.8 M LiCl for 2 h. The radioactivity was detected with a Bio-Image Analyzer (FLA2000, Fuji, Tokyo, Japan).

To prepare the 3′-labelled helicase substrate, unlabelled 37-mer (3 pmol) annealed to M13mp18 single-stranded DNA was labelled at the 3′-OH in the reaction mixture (180 μl) consisting of 25 units of DNA polymerase I Klenow fragment, 1.6 μM [α-^{32}P]dCTP, 0.1 mM dGTP, 1 mM dideoxy-TTP, 15 mM Tris–HCl (pH 7.9), 7.5 mM $MgCl_2$ and 1 mM DTT. The mixture was

incubated for 20 min at 30°C and then for an additional 15 min with a further addition of unlabelled dCTP to the final concentration of 50 μM in order to prepare 3′-labelled 40-mer oligonucleotide annealed to M13 single-stranded DNA. The 5′-labelled helicase substrate was prepared as described above. DNA helicase substrates to examine the directionality of the translocation were prepared as follows. A total of 0.5 pmol of the 5′-labelled helicase substrate (37-mer oligonucleotide) or the 3′-labelled helicase substrate (40-mer oligonucleotide) was digested with *Sma*I. The aliquots (2–2.5 fmol) of the digested DNA were used for the DNA helicase assay, as described above.

UV-Mediated Crosslinking of ATP—The mixture in a final volume of 20 μl contained 20 mM HEPES buffer (pH 7.5), 10% glycerol, 0.1 mM DTT and 5 μCi of [α-^{32}P]ATP (3000 Ci/mmol), as well as 0.25 or 0.5 μg of hRECQL4 protein and 0.5 μg of MCM4/6/7 complex, or *Escherichia coli* DNA polymerase I Klenow fragment (two units, Toyobo, Japan). When DNA polymerase I was incubated, non-radioactive ATP was added to the reaction at a final concentration of 5 μM. After the mixture was incubated for 10 min on ice, UV irradiation (254 nm) was carried out in a microcentrifugation tube at 4°C for 30 min at a distance of 5 cm (Mineralight, UVP, Upland, CA, USA). Upon termination of UV exposure, 0.8 μl of 100 mM ATP and 10 μg of bovine serum albumin were added to the mixture. Trichloroacetic acid was added to a final concentration of 10%, and the mixture was incubated for 10 min on ice. The precipitate was recovered by centrifugation; it was then washed once with acetone containing 0.5% hydrochloric acid and then twice with acetone alone. Proteins were separated by SDS–polyacrylamide gel electrophoresis (10%), and labelled proteins were visualized by using the Bio-Image Analyzer.

Others—SV40 T antigen was purified as reported (*15*). Human RPA complex was prepared as described below. Human *RPA1*, *RPA2* and *RPA3* cDNA synthesized from mRNA of HeLa cells by RT–PCR method (Invitrogen, Carlsbad, CA) were cloned into baculovirus vectors, pVL1393, pAcUW31 and pVL1393, respectively. *RPA1* was cloned to be expressed as a $(his)_6$-RPA1 fusion protein, and *RPA2* was as a flag-RPA2 fusion protein. High5 cells were co-infected with the three viruses expressing RPA1, 2 and 3 proteins for 2 days. The recombinant RPA proteins in infected cell lysate were purified by Ni-nitrilotriacetic acid (NTA) (Qiagen, Hilden, Germany) affinity column chromatography as follows. The infected cells were suspended in lysis buffer consisting of 10 mM Tris–HCl (pH 7.5), 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM Na phosphate buffer, 10 mM $Na_4P_2O_7$ and protease inhibitors (Pharmingen, BD, San Jose, CA). After incubation for 40 min on ice, insoluble material was removed by centrifugation at 40,000 rpm (TLS55, Beckman, Fullerton, CA) for 40 min at 4°C. To one volume of the clarified lysate, one-tenth volume of Ni–NTA–agarose was added, and the mixture was incubated for 1 h at 4°C on a rocking platform. The beads were then collected by centrifugation and stringently washed with buffer A (50 mM Na–phosphate buffer, pH 6.0, 300 mM NaCl and 10% glycerol) containing 20 mM imidazole. Next the beads were washed once with buffer B (50 mM Na–phosphate buffer, pH 8.0, 300 mM NaCl and 10% glycerol) containing 20 mM imidazole, and the proteins bound to the beads were eluted by adding one bead volume of buffer B containing 300 mM imidazole. This was followed by incubation for 5 min at 4°C on a rocking platform and removal of the beads by centrifugation. The elution of proteins was repeated two more times. The pooled eluates were diluted to decrease NaCl concentration to 50 mM and concentrated by Centricon 30 (Millipore, Bedford, MA). The concentrated proteins were loaded on a MonoQ column, and the bound proteins were eluted using a linear 0.1–0.6 M NaCl gradient. All the three RPA1, 2 and 3 proteins were co-eluted with about 0.3 M NaCl and they were concentrated using Microcon 30 after the salt concentration was decreased to 0.1 M.

RESULTS

Cloning and Expression of hRECQL4 Gene—Human *RECQL4* cDNA was synthesized from the mRNA of HeLa cells, and the gene was amplified by the RT–PCR method. Since it was difficult to amplify the gene, PCR was performed in the presence of betain, which will prevent the formation of the secondary structure of template DNA (*13*). The amplified DNA was cloned into pcDNA 3.1 DNA. Several clones have been isolated, but the sizes of the inserted DNA in the clones were different in size (data not shown). To select a clone containing full-size human *RECQL4* gene, proteins were synthesized from the cloned DNAs by a transcription-coupled translation system using rabbit reticulocyte lysate. Approximately 150 kDa of protein was synthesized from one cloned DNA. Inserted DNA of the plasmid was recloned into a baculovirus vector; its nucleotide sequence was then analysed. The nucleotide sequence of the DNA was compared to that of the human *RECQL4* gene (NM_004260) banked in the National Center for Biotechnology Information (NCBI). Three nucleotides (nucleotides 753, 801 and 1772) in the cloned DNA were different from those in the banked gene; as a result, differences in two amino acids (amino acids 267 and 591) of a total of 1,208 amino acids were detected between two proteins (Fig. 1A). One is located in the amino-terminal half of the hRECQL4 protein and another is within the helicase domain. Since the latter is not located in the conserved ATP-binding motifs, we produced a protein from this clone.

Recombinant baculoviruses were prepared using the cloned DNA, and a protein of approximately 150 kDa protein was detected with anti-flag antibody in a lysate of infected insect cells, but not in one of uninfected cells (Fig. 1B). hRECQL4 protein in the lysate was purified using anti-flag antibody agarose. After binding to the agarose beads, the flag-hRECQL4 protein was detached from the beads by incubating with a buffer containing flag peptides. The eluate was further fractionated by glycerol gradient centrifugation (Fig. 2A). A hRECQL4 protein of about 150 kDa mainly sedimented at a position between catalase (232 kDa) and ferritin (440 kDa), suggesting that hRECQL4 protein forms homo-multimers.

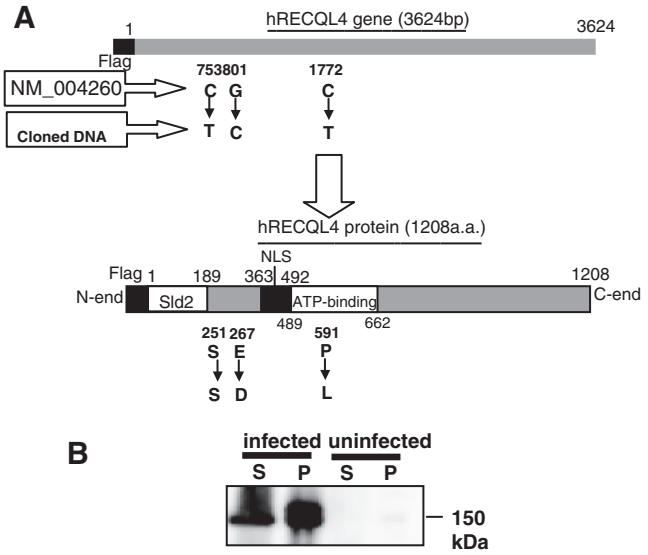

Fig. 1. **Sequence of cloned *hRECQL4* gene.** (A) Cloned human *RECQL4* gene attached with nucleotides from flag peptide at the amino-terminus was sequenced. At the top, the sequence is compared to the published human *RECQL4* gene (NM_004260); nucleotides that differed are indicated with the nucleotide position of the published gene. At the bottom, the primary structure of the hRECQL4 protein with an amino-terminal flag tag, a conserved ATP-binding domain, a region homologous to Sld2 from *S. cerevisiae* and nuclear localizing signal (NLS) (*29*) are shown; changes in amino-acids resulting from the differences in nucleotides are indicated. (B) Sf9 cells were infected with recombinant baculovirus for expression of hRECQL4 protein. Proteins in Triton-soluble (S) and -insoluble (P) fractions from infected and uninfected cells were examined by immuno-blotting using anti-flag antibody.

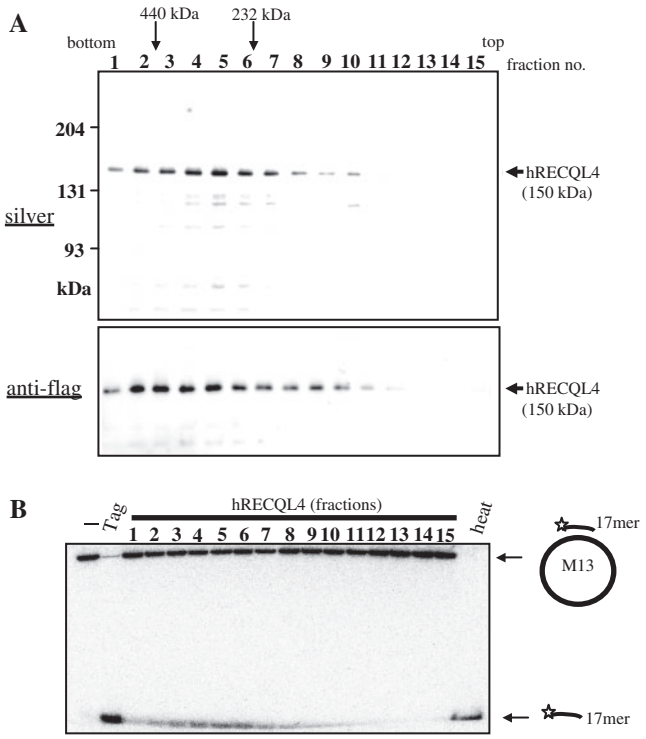

Fig. 2. **Glycerol gradient centrifugation of hRECQL4 protein.** (A) hRECQL4 protein purified from baculovirus-infected cell lysate with anti-flag antibody agarose was further purified by glycerol gradient centrifugation. The proteins in the recovered fractions were analysed in 8% acrylamide gel containing SDS. They were stained with silver (top) and they were examined by immuno-blotting with anti-flag antibody (bottom). The positions where catalase (232 kDa) and ferritin (440 kDa) sedimented are indicated. (B) DNA helicase activity that displaced 17-mer oligonucleotide annealed to single-stranded circular DNA was measured in the presence of ATP. As controls, the substrate DNA incubated with SV40 T antigen (Tag) and heat-denatured substrate DNA (heat) were electrophoresed.

Since the hRECQL4 protein was purified to near homogeneity, an aliquot of the glycerol gradient fractions was assayed for DNA helicase activity that displaces 17-mer oligonucleotide annealed to single-stranded circular DNA in the presence of ATP (Fig. 2B). The oligonucleotide-displacing activity was detected in the fractions where hRECQL4 protein sedimented. The amounts of hRECQL4 protein in the fractions appear to be proportionate to the level of DNA helicase activity, suggesting that hRECQL4 protein exhibits the activity.

To further address the relationship between hRECQL4 protein and DNA helicase activity, the protein purified with the anti-flag antibody agarose was purified via anion-exchange ion column chromatography using MonoQ (Fig. 3A). hRECQL4 protein was eluted with about 0.3 M of NaCl. DNA helicase activity was co-eluted with the hRECQL4 protein (Fig. 3B) and the activity to hydrolyse ATP was also co-eluted with the protein (Fig. 3C). Since the ATP-hydrolysing activity was not detected in the absence of single-stranded DNA, the activity is single-stranded DNA-dependent. Similar results were obtained by the purification using MonoS column (data not presented). In order to examine the interaction of hRECQL4 protein and ATP, the purified protein was incubated with labelled ATP, and ATP bound with hRECQL4 was cross-linked by UV irradiation. The products were analysed by SDS–polyacrylamide gel electrophoresis (Fig. 4). As positive controls, a DNA polymerase I Klenow fragment from *E. coli* and human MCM4/6/7 complex were used in this experiment. A strong 70-kDa signal from the Klenow fragment and a weak 100-kDa signal from the hMCM6 protein in the complex were detected (*14*). A signal of about 150 kDa was detected in the reaction containing hRECQL4 protein, suggesting that hRECQL4 interacts with ATP. All these results suggest that hRECQL4 protein exhibits DNA helicase activity.

Characterization of DNA Helicase Activity—First, the pH dependency of DNA helicase activity in the purified hRECQL4 protein was examined. The relatively higher activity was detected at a pH range of 8–10 (Fig. 5A). The DNA helicase activity in purified hRECQL4 protein was able to displace 17-mer oligonucleotide annealed to circular single-stranded DNA, but it could not displace annealed 37-mer and 53-mer oligonucleotide under comparable conditions (Fig. 5B), suggesting that the processibility of the DNA helicase is low. To examine the directionality of movement on the single-stranded DNA, two DNA substrates were prepared. The DNA helicase

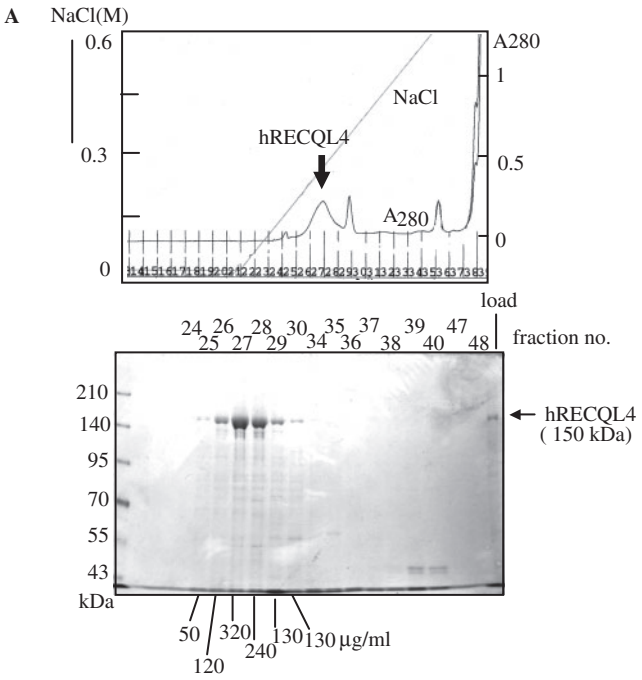

Fig. 3. **MonoQ column chromatography of hRECQL4 protein.** (A) hRECQL4 protein purified using anti-flag antibody agarose was further fractionated by MonoQ column chromatography. Proteins were eluted from the column by a linear NaCl gradient from 0 M to 1 M. Protein profile detected by the absorbance at 280 nm as well as a profile of the NaCl gradient, are shown. A position where hRECQL4 protein elutes is indicated by an arrow (top). Proteins eluted from the MonoQ column were analysed in 8% acrylamide gel containing SDS, and they were stained with coomassie brilliant blue (bottom). Protein concentration in the fractions was measured using a dye (Bio-Rad, Hercules, CA, USA), and they are indicated at the bottom. (B) DNA helicase activity in the MonoQ fractions (nos. 24–34) was measured where 0.2 μl of the fractions was added to the reaction. (C) ATP-hydrolysing activity in the fractions was measured in the absence or presence of single-stranded DNA as indicated. A total of 2 μl of the fractions was added to the reaction. Released phosphate (Pi) was detected via thin layer chromatography.

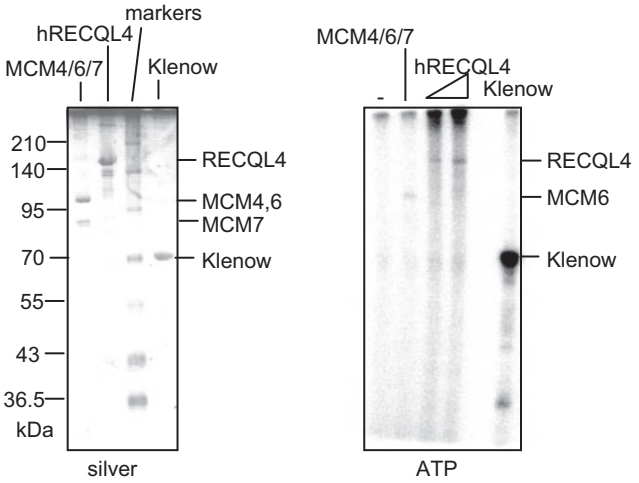

Fig. 4. **UV crosslinking of hRECQL4 and ATP.** hRECQL4 protein from MonoQ fraction no. 28 (0.24 and 0.48 μg), human MCM4/6/7 complex (0.5 μg), and DNA polymerase I Klenow fragment (two units) from *E. coli* were incubated with [α-^{32}P] ATP, and proteins bound with ATP were crosslinked by UV irradiation. The protein-ATP complexes were analysed by SDS–polyacrylamide gel electrophoresis (10%) and autoradiography was performed (right). hRECQL4 protein (0.19 μg), human MCM4/6/7 complex (0.22 μg), and DNA polymerase I Klenow fragment (0.8 units) were electrophoresed with marker proteins and stained with silver (left).

displaced only 5′-labelled 18-mer, not 3′-labelled 22-mer; this suggests that it migrates on single-stranded DNA in a 3′–5′ direction. As a positive control of the displacement of 3′-labelled 22-mer, human RPA, a single-stranded DNA-binding protein, was added to the reaction (*16*).

The ATP requirement of the DNA helicase activity was examined (Fig. 6). The helicase activity was detected in the presence of ATP, but not detected in the presence of non-hyrolysable-type ATP analogues (ATPγS and AMPPNP) or in the absence of ATP (Fig. 6A). Thus, the DNA helicase activity is dependent on the presence of hydrolysable ATP. It is known that ATP complexes with Mg ions; the concentration of ATP and $MgCl_2$, instead of $Mg(OAc)_2$ in the standard reaction, was titrated in the DNA helicase reaction containing hRECQL4 protein. The helicase activity was optimal in the presence of 10–15 mM ATP/$MgCl_2$ (Fig. 6B). To further understand the requirements of ATP and Mg for DNA helicase activity, DNA helicase activity in the purified hRECQL4 protein was measured in the reactions having different concentrations of ATP and a fixed concentration (5 mM) of $MgCl_2$ (Fig. 6C, left) or in the reactions having different concentrations of $MgCl_2$ and a fixed concentration (5 mM) of ATP (Fig. 6C, right). In the reactions containing 5 mM $MgCl_2$, the activity

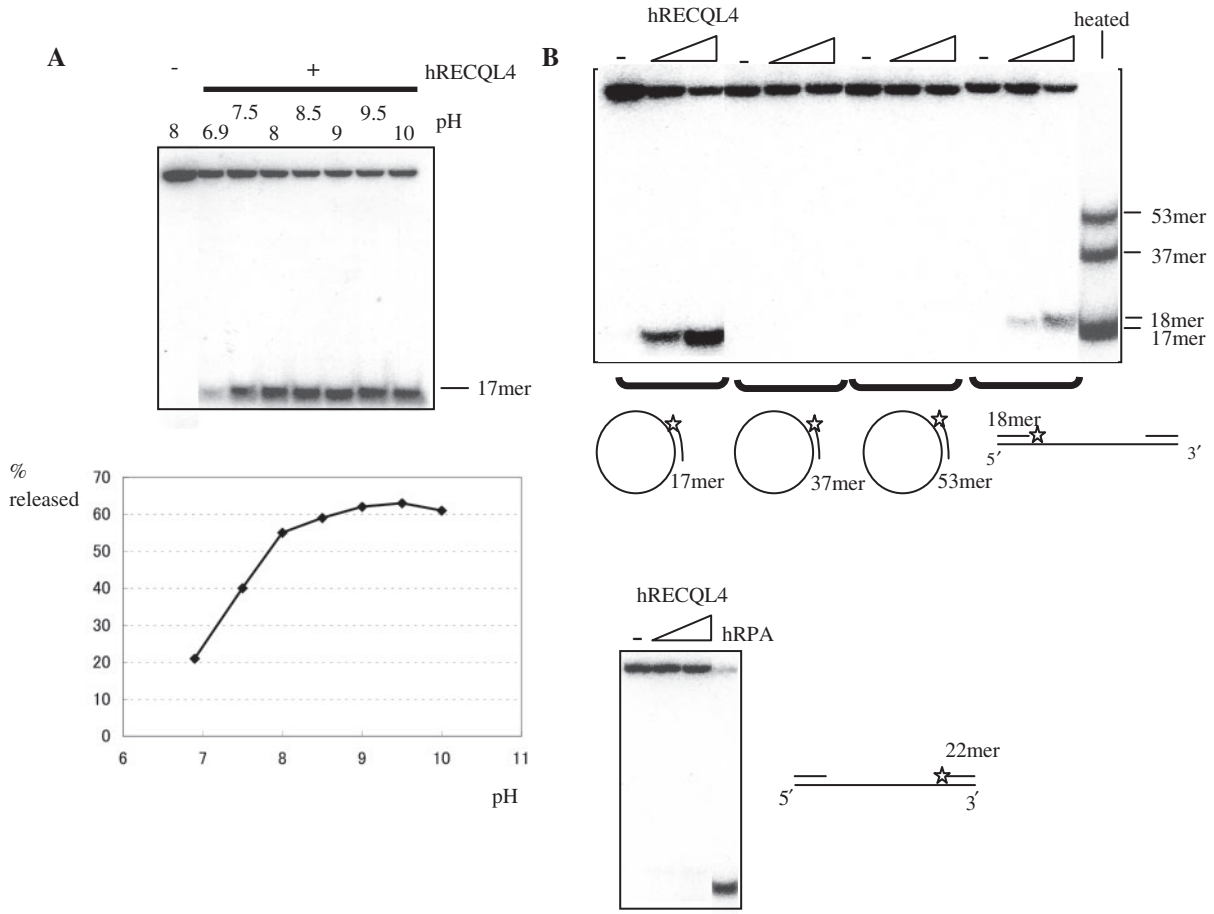

Fig. 5. **Characterization of DNA helicase activity.** (A) The pH-dependency of DNA helicase activity was examined. The hRECQL4 protein was purified with anti-flag antibody agarose and then by glycerol gradient centrifugation. The activity of the purified protein (52 ng) was measured in reactions containing Tris buffer with different pHs. At the bottom, the proportion of released 17-mer in these reactions is calculated. (B) DNA helicase activity in the purified fraction was measured using DNA substrates with different sizes of oligomers, as indicated. Increasing amounts (35 and 70 ng) of hRECQL4 protein were added to the reactions. To determine the directionality of movement on the single-stranded DNA, two substrates were used in the DNA helicase reaction. One is 5′-labelled 18-mer, annealed to single-stranded linear DNA (top); the other is 3′-labelled 22-mer, annealed to the DNA (bottom). As a positive control for the latter reaction, hRPA, a human single-stranded DNA-binding protein was included in the DNA helicase reaction.

was almost undetectable in the absence of ATP. The activity was maximal in the presence 2.5–5 mM ATP and it was slightly decreased in the presence of 7.5–10 mM ATP. In the reactions containing 5 mM ATP, the activity was undetectable in the absence of $MgCl_2$ and gradually increased in proportion to the concentration of $MgCl_2$. These results indicate that the DNA helicase activity is dependent on the presence of ATP and $MgCl_2$ and the activity is stimulated in the conditions where the concentration of $MgCl_2$ was higher than that of ATP.

Next, in order to understand the requirements of ATP and Mg for ATPase activity of purified hRECQL4 protein, the concentration of ATP and $MgCl_2$ was titrated in the ATPase reaction by hRECQL4 protein (Supplementary Fig. 1S-A). The ATPase activity was almost undetectable in the absence of ATP and $MgCl_2$. The activity was detected in the presence of 2.5 mM ATP and $MgCl_2$, and it was almost unchanged in the reactions containing 2.5–20 mM ATP and $MgCl_2$. To further understand the requirements of ATP and Mg, the ATPase activity in the purified hRECQL4 protein was measured in the reactions having different concentrations of ATP and a fixed concentration (5 mM) of $MgCl_2$ (Supplementary Fig. 1S-B, left) or in the reactions having different concentrations of $MgCl_2$ and a fixed concentration (5 mM) of ATP (Supplementary Fig. 1S-B, right). In the reactions containing 5 mM $MgCl_2$, the activity was detected even in the absence of ATP and it was almost unchanged in the reactions containing 2.5–10 mM ATP. In the reactions containing 5 mM ATP, the activity was undetectable in the absence of $MgCl_2$. The activity was detected in the presence of 2.5 mM $MgCl_2$ and it was almost unchanged in the reactions containing 2.5–10 mM $MgCl_2$. These results indicate that the ATPase activity in the purified hRECQL4 proteins is dependent on the presence $MgCl_2$ and it requires relatively lower concentrations of $MgCl_2$ than the DNA helicase activity.

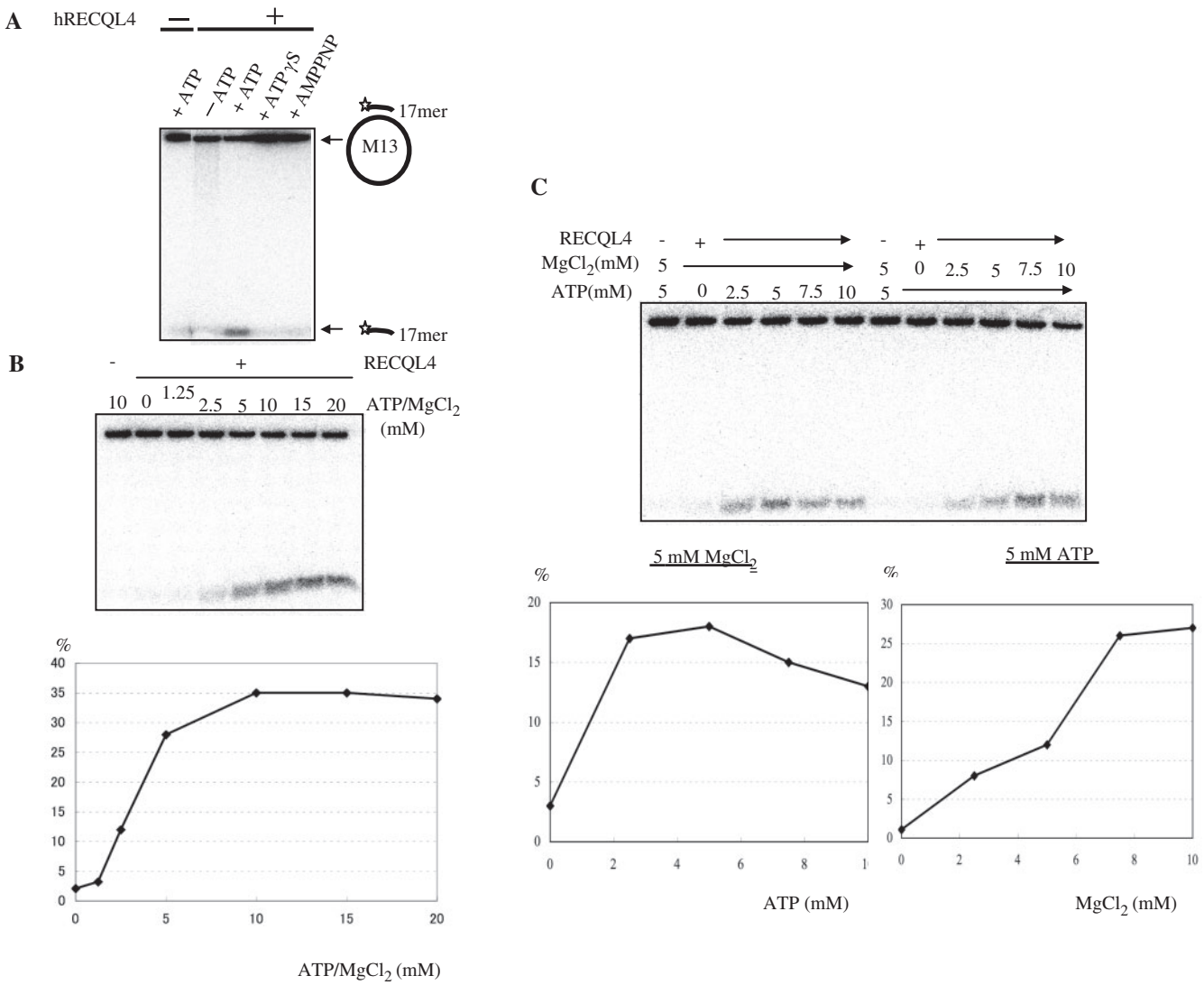

Fig. 6. **ATP and Mg requirement of DNA helicase activity.** (A) DNA helicase activity in the hRECQL4 protein purified by glycerol gradient centrifugation was measured in the presence of ATP or non-hydrolysable-type ATP analogues (ATPγS and ANPPNP), or in their absence. (B) DNA helicase activity in the hRECQL4 protein purified by MonoS column chromatography was measured in the reactions having different concentrations of ATP and $MgCl_2$. Equal concentrations of ATP and $MgCl_2$ were added. At the bottom, the proportion of released 17-mer was calculated and its highest value among the reactions was rendered to be 100%. (C) DNA helicase activity in the purified hRECQL4 protein was measured in the reactions having different concentrations of ATP and a fixed concentration (5 mM) of $MgCl_2$ (left) or in the reactions having different concentrations of $MgCl_2$ with a fixed concentration (5 mM) of ATP (right). At the bottom, the proportion of released 17-mer was calculated.

DISCUSSION

In this article, it has been shown that DNA helicase activity and a single-stranded DNA-dependent ATP hydrolysing activity are co-purified with recombinant human RECQL4 protein. The results showing co-sedimentation of the DNA helicase activity and hRECQL4 protein in glycerol gradient centrifugation as well as the co-elution of these two from the MonoQ column strongly suggest that the hRECQL4 protein exhibits DNA helicase activity. The direct interaction of hRECQL4 protein with ATP in UV-mediated cross-linking experiments supports this conclusion. However, it remains to be determined, whether the activity is intrinsic to hRECQL4 protein or derived from contaminating other proteins. The effect of an amino-acid change at a site of 591 on the DNA helicase activity should also be addressed.

Human RECQL1 (*4*, *5*), 2, 3 and 5β (*6*) proteins all exhibit DNA helicase activity *in vitro*. It has been reported that human RECQL4 protein, which is expressed in *E. coli* (*17*) or immunoprecipitated from HeLa cells (*18*), exhibits ATP-hydrolysing activity but not DNA helicase activity. Although the apparent discrepancy between these findings and the present data remains to be explored, following differences in DNA helicase reactions can be pointed out. First, ATP and Mg concentrations in the published papers (*17*, *18*) may

be too low. The results in Fig. 6 indicate that the DNA helicase activity of the purified hRECQL4 protein requires relatively higher concentrations of ATP and Mg. In contrast, as shown in Supplementary Fig. 1S, the ATPase activity requires lower concentrations of ATP and Mg and the activity was detected even in the absence of ATP. Second, the amounts of DNA substrates in the published papers (*17*, *18*) are 10–50-fold higher than those in the present system. The single-stranded DNA region in the substrates may trap RECQL4 proteins. Third, the size (40-mer) of DNA duplex region in the paper (*18*) may be too long to be displaced from the results in Fig. 5B. Recently, it has been reported that hRECQL4 prepared from overexpressed *E. coli* exhibits DNA helicase activity *in vitro* in the reaction mixtures containing 5 mM of ATP and Mg (*19*). Excess amounts of one strand of the helicase substrate are required to detect the DNA helicase activity, suggesting that an annealing activity of hRECQL4 protein may mask the DNA helicase activity under standerd conditions (*19*). It remains to be determined whether the purified hRECQL4 protein in this study exhibits the annealing activity. In the published paper (*19*), it has been shown that two distinct regions of hRECQL4 protein, the conserved helicase mitifs and the Sld2-like N-terminal domain, each independently promote ATP-dependent DNA unwinding.

Based on the information in this article and the results from published paper on other hRECQL proteins, it is suggested that the 3′–5′ directionality of migration on single-stranded DNA is common among the five human RECQL members. The requirement of a high concentration of ATP and $MgCl_2$ is a unique feature of the DNA helicase activity in the purified hRECQL4 protein among the members (*5*, *20*). *In vitro* nuclear DNA-replication systems require 5–10 mM ATP for the maximal level of DNA-replication activity (*21–25*). Such a high concentration of ATP is probably required for DNA helicase that is responsible for the unwinding of the DNA-replication forks. Our group observed that MCM4/6/7 helicase required 10–15 mM ATP for the maximal level of the activity (Ishimi *et al*., unpublished results); thus, due to the requirement of a high concentration of ATP, both the RECQL4 protein and MCM4/6/7 complex are good candidates for replicative DNA helicase.

From the results of Sangrithl *et al*. (*9*), it is suggested that *Xenopus* RecQL4 protein functions as a DNA helicase at the initiation of DNA replication; our results are consistent with this notion. It is also probable that the DNA helicase activity of RECQL4 protein is required for the regulation of DNA recombination, to maintain genome stability *in vivo* (*26–28*). The carboxyl-terminal portion including the conserved DNA helicase domains is absent from the mutant RECQL4 proteins of patients with Rothmund–Thomson syndrome (*7*), suggesting that the helicase domains are dispensable in terms of maintaining human viability. However, severe growth retardation was detected in helicase activity-inhibited mouse (*29*). The amino-terminal region of hRECQL4, which is homologous to Sld2 protein in *S. cerevisiae*, may play an indispensable role in viability. It remains to be determined, whether the amino-terminal region interacts with the GINS complex to assemble the complex in the replication initiation region. The finding that the hRECQL4 protein forms multimer may be important in terms of its presumable Sld2-like function as well as the structure and function of DNA helicase activity.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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CONFLICT OF INTEREST

None declared.

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