Drosophila melanogaster RECQ5/QE DNA helicase: stimulation by GTP binding

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ABSTRACT

The Drosophila melanogaster RECQ5/QE gene encodes a member of the DNA helicase family comprising the Escherichia coli RecQ protein and products of the human Bloom’s, Werner’s, and Rothmund-Thomson syndrome genes. The full-length product of RECQ5/QE was expressed in the baculovirus system and was purified. Gel filtration experiments indicated that RECQ5/QE was present in an oligomeric state. The RECQ5/QE protein hydrolyzed ATP and even more actively GTP in the presence of single-stranded DNA. ATP drove the DNA helicase activity of RECQ5/QE, whereas GTP had little effect. GTP exhibited a stimulatory effect on DNA unwinding when it was used together with ATP. This effect was more apparent with non-hydrolyzable GTP analogs, such as GTPγS and GMPPNP. These results indicate that GTP binding to RECQ5/QE triggers its DNA helicase activity. GTP binding increased the rate of strand separation without affecting the $S_{0.5}$ ($K_m$) values for the substrates during the DNA helicase reaction. The data collectively suggest that the RECQ5/QE protein is activated upon GTP binding through the ATP-binding site.

INTRODUCTION

Helicases are ubiquitous enzymes involved in almost all aspects of nucleic acid metabolic pathways. Separation of double-stranded DNA (dsDNA) or base-paired regions in single-stranded DNA (ssDNA) molecules is a prerequisite for basic genetic processes, such as genome replication, repair, recombination and expression at multiple stages. The loss of basic genetic processes, such as genome replication, repair, and expression at multiple stages. The loss of helicase function causes a number of disorders in organisms. Three hereditary disorders (Werner’s syndrome, Bloom’s syndrome and a subset of Rothmund-Thomson syndrome) in humans are associated with the loss of function of the respective RecQ homologs [BLM, WRN and RTS (1–3)]. The eukaryotic RecQ homologs are thus named due to their similarity to the Escherichia coli RecQ helicase, which participates in the bacterial RecF genetic recombination pathway (4). The RecQ helicase domains of BLM, WRN and RTS are similar to those of the E.coli RecQ enzyme. However, the mammalian proteins are larger, due to the presence of additional flanking domains. To date, five RecQ homologs have been identified in the human genome [RecQ1/QL, BLM, WRN, RTS and RecQ5 (5–7)].

In contrast to BLM, WRN and RTS, RecQ1/QL and RecQ5 possess only short N-terminal regions preceding the helicase domain. The functions of these short N-terminal type RecQ homologs are hitherto unknown. Recently, we isolated a Drosophila RecQ5 homolog, RECQ5/QE, specifically expressed in early embryos (8). The results of the Drosophila Genome project revealed that Drosophila melanogaster contains three RecQ homologs, Blm, Rts and RECQ5/QE, in addition to a Werner exonuclease protein family member (9,10). The only short N-terminal-type RecQ protein in D.melanogaster appears to be RECQ5/QE, since no RecQ1/QL homolog has been identified so far. In both humans and Drosophila, RecQ5 exists as three isoforms produced by alternative splicing (11). Small isoforms of human RecQ5 localize to the cytoplasm, while the large isoform is nuclear (12). The RECQ5/QE protein isolated from Drosophila by our group represents the large isoform, and localizes to the nucleus (S. Maruyama and K. Kawasaki, unpublished results; 11).

The predicted RECQ5/QE gene product is a 1058 amino acid protein that contains a helicase domain comprising seven helicase motifs. We propose that RECQ5/QE encodes an active helicase, based on the results of studies on the in vitro transatlational product (8). In addition, a small RECQ5 isoform may function as a 3’ to 5’ DNA helicase (13). The presence of multiple RecQ homologs in a single organism suggests that each enzyme has a distinct role (7). Therefore, to elucidate the mechanistic and functional characteristics of RECQ5/QE, it is necessary to determine the biochemical properties of the protein. To achieve this goal, we have employed an insect-based expression system that permits the preparation of homogeneous RECQ5/QE protein with a reasonable yield.

We report here that the RECQ5/QE protein is a ssDNA-stimulated ATPase and an ATP-dependent DNA helicase.
Furthermore, we demonstrate that RECQ5/QE is a ssDNA-dependent GTPase, and that GTP binding stimulates the ATP-dependent DNA helicase activity of the protein.

MATERIALS AND METHODS
Construction of BmNPV–RECQ5/QE

The full-length Dral–NcoI RECQ5/QE cDNA (−16 to +3487) was cloned into the SmaI–NcoI sites of the pBm31 transfer vector under the control of the polyhedrin promoter. A recombinant virus, BmNPV–RECQ5/QE, was generated by homologous recombination in BmN cells by co-transfection with polyhedrin-deficient Bombyx mori nucleopolyhedrovirus (BmNPV-abb) (14).

Purification of the RECQ5/QE protein

Typically, 26 plates (150 mm dishes) of BmNPV–RECQ5/QE-infected BmN cells were washed with cold phosphate-buffered saline (PBS), harvested, and stored at −80°C, 2 days after infection. Infected cells (6.7 g) were suspended in 33 ml (5 ml/g cell) of lysis buffer [50 mM Tris–HCl, pH 8.5, 10 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Nonidet P-40 (Calbiochem) and protease inhibitor cocktail (Complete; Roche)]. After 30 min on ice, the lysate was centrifuged at 10,000 g for 10 min. The pellet was extracted with 0.45 M KCl in lysis buffer for 30 min on ice, and was subjected to further centrifugation. The majority of the RECQ5/QE protein was recovered in the supernatant, which was further diluted to an electronic conductivity equivalent to 0.3 M KCl with Buffer Q (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10% glycerol), and was subjected to chromatography on DEAE–Sephacel (11 ml; Pharmacia). The flow-through fraction from DEAE–Sephacel was diluted to a KCl concentration of 0.1 M, and was loaded onto a heparin–Sephrose CL6B column (7 ml; Pharmacia) pre-equilibrated with 0.1 M KCl in Buffer Q. A linear KCl gradient (0.1–1.0 M KCl) was used for elution. The RECQ5/QE protein eluted at 0.44 M KCl, as determined by SDS–polyacrylamide gel electrophoresis (PAGE) of fractions and immunoblotting with anti-RECQ5/QE antibodies (8). The DNA-dependent ATPase and GTPase activities were measured. The active fraction (8 ml) was loaded onto a Mono S column (1 ml HR5/5; Pharmacia) pre-equilibrated with 20 mM Tris–HCl, pH 7.5, 0.1 mM NaCl, 0.5 mM EDTA and 1 mM DTT, and was eluted using 10 vol of a linear NaCl gradient (0.1–1.0 M) in the same buffer. The RECQ5/QE protein eluted at ~0.35 M NaCl. The purified, active protein was stored at −70°C. The heparin–Sephacel fraction was also analyzed using Superose 6 (HR10/30; Pharmacia) pre-equilibrated with 50 mM HEPES, pH 7.5, 300 mM KCl, 1 mM DTT and 1 mM EDTA. The protein concentration was determined with a BioRad protein assay kit, using bovine serum albumin as the standard.

Preparation of extracts from Drosophila Schneider cells

Drosophila Schneider S2 cells were harvested from a confluent 100 mm dish. The collected cells were washed once with PBS and were resuspended in 0.5 ml of 20 mM HEPES–NaOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 1 mM DTT, and protease inhibitor cocktail. After 20 min on ice, the DNA was sheared through a 25G needle. The suspension was centrifuged at 10,000 g for 20 min. The supernatant was filtered through a 0.45 μm membrane. A 0.2 ml aliquot of supernatant was loaded on the Superose 6 column.

DNA-dependent ATPase and GTPase assays

DNA-dependent ATPase assays were performed as described previously (15). The reaction mixture (20 μl), containing 20 mM Tris–HCl, pH 7.5, 2 mM DTT, 90 μg/ml bovine serum albumin, 50 μM [nucleotides (nt)] M13mp18 virion DNA and 1.3 mM [35S]Mg2+-ATP (14 MBq/nmol; Amersham), was incubated at 27°C for 30 min. For the measurement of the GTPase activity, 2.6 mM [35S]Mg2+-GTP (360 MBq/nmol; NEN) was employed, instead of labeled ATP. The reaction was stopped by the addition of 10 μl of 3 mM ATP, ADP, AMP, and 25 mM EDTA on ice. Aliquots were spotted onto a polyethyleneimine sheet (Polygram CEL300PEI; Macherey-Nagel) and were developed in 0.5 M LiCl, 1 M HCOOH. Radioactivity was quantitated using a BAS2500 Imaging plate reader (Fuji).

Preparation of helicase substrates

A 69mer (5′-CCA AGC TTG CAT GCC TGG ACT CTA GAG CAT CCC CGG GTA CCG AGC TCG AAT-3′) was labeled with [γ-32P]ATP using T4 polynucleotide kinase (TaKaRa), and was purified by Sephadex G-50 chromatography (Probe-Quant; Pharmacia). The labeled oligomer was annealed to M13 mp18 virion DNA. Alternatively, a 17mer (5′-GTAAGACCGACGCCAGT-3′) was annealed to M13mp18 virion DNA and was labeled with [α-32P]dCTP and dGTP to produce a 20mer, using Klenow fragment (TaKaRa). The labeled product was purified by Sephadex G-50 chromatography. Next, the annealed substrates were purified by Sepharose CL6B gel filtration.

For the determination of polarity, a 48mer (5′-GTG CCA AGC TTG CAT GCC TGG ACT CTA GAG CAT CCC CGG GTA-3′) was labeled with [α-32P]dCTP using Klenow fragment after annealing to M13mp18 virion DNA, or with [γ-32P]ATP using T4 polynucleotide kinase before annealing to virion DNA. After purification using Sephadex G-50 chromatography, the annealed substrates were digested with HincII and were purified by Sepharose CL6B gel filtration.

Helicase assays

Helicase assays measure strand displacement activity, where- by a partially dsDNA substrate is converted to its component single-strand products. The helicase substrate, 1.0 μM (nt) M13mp18 ssDNA annealed with 32P-labeled oligomer (20mer or 69mer), was incubated with purified RECQ5/QE in a reaction mixture (20 μl) comprising 20 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, 2 mM DTT, 90 μg/ml bovine serum albumin, 2 mM MgCl2 and 2 mM ATP. After an incubation at 27°C for 10 min, the reaction was stopped by the addition of 5 μl of 75 mM EDTA, 5% Sarkosyl, 0.1% Bromophenol blue, 30% glycerol, and 2 μl of phenol/chloroform (1:1) on ice. The reaction products were separated on a 1% agarose gel (for the 69mer-labeled oligonucleotide) in buffer containing 40 mM Tris acetate, pH 8.0, 1 mM EDTA, as described elsewhere (16), or on a 12% polyacrylamide gel (for the 20mer-labeled...
oligonucleotide) in buffer containing 89 mM Tris borate, pH 8.3, 2 mM EDTA. Polyacrylamide gels were dried on Whatman DE81 paper and agarose gels were dried on GelBond PAG film (BMA). Dried gels were analyzed using the BAS 2500 Imaging plate reader, or were exposed to X-ray film for autoradiography. Experiments were repeated at least twice. The results were reproducible with little gel-to-gel variation, thus allowing quantitation among samples on different gels. Quantitative data from a single gel are shown.

SDS–PAGE, immunoblotting and silver staining
SDS–PAGE was performed according to the procedure of Laemmli (17). Rainbow molecular weight markers (Amersham) or prestained molecular weight markers (NEB) were used as molecular weight standards. Immunoblotting was performed as described previously (8). Silver staining of the gel was achieved using the Silver Stain Plus kit (BioRad).

RESULTS
Purification of full-length RECQ5/QE protein
Initial attempts to purify RECQ5/QE expressed in E. coli were unsuccessful because of the poor expression and the insolubility of the protein. To circumvent these problems, the recombinant virus, BmNPV–RECQ5/QE, containing the full-length RECQ5/QE under the control of the BmNPV polyhedrin promoter, was generated (Materials and Methods). Cells infected with BmNPV–RECQ5/QE yielded a soluble product that migrated on SDS–PAGE with an apparent molecular mass of 120 kDa. This corresponded to the expected molecular mass determined from the open reading frame of RECQ5/QE (9). Immunoblotting of crude lysates of infected cells with anti-RECQ5/QE antibodies revealed a single 120 kDa band, confirming that the overexpressed protein was the desired product. The majority of the RECQ5/QE protein was recovered from a 0.45 M KCl extract and not the cytosol, suggesting nuclear localization. The protein was not detected in BmN cells prior to infection or by mock infection (data not shown). The 120 kDa protein was purified to near homogeneity by successive chromatography steps, as monitored by immunoblotting and DNA-dependent ATPase and GTPase assays (Materials and Methods; Fig. 1A).

The RECQ5/QE protein is oligomeric
We examined the native molecular mass of the RECQ5/QE protein. Superose 6 gel filtration of the RECQ5/QE protein in the presence of 0.3 M KCl revealed a peak at 480 kDa (Fig. 1B, C and E). This apparent molecular mass corresponds to a tetramer of the 120 kDa RECQ5/QE protein. We did not detect RECQ5/QE in smaller molecular weight fractions corresponding to monomers or dimers. The RECQ5/QE protein in cultured cells showed the same molecular mass (Fig. 1D), suggesting that the protein exists in an oligomeric form.

The RECQ5/QE protein is a DNA-dependent ATPase
‘Walker Box’ motifs, which predict ATPase activity, were observed in the primary sequence of RECQ5/QE. The ability of the RECQ5/QE protein to hydrolyze ATP was therefore examined. Figure 2 shows that RECQ5/QE is associated with an ATPase activity that is strongly dependent on the presence of ssDNA. The substrate turnover rate was calculated as 18.2 s⁻¹ with M13mp18 virion DNA as a co-factor, and 3.23 s⁻¹ without DNA. Moreover, the ATPase activity required divalent ions. Mg²⁺ could be replaced by Mn²⁺ or Ca²⁺, but
RECQ5/QE DNA helicase has low processivity. Since blunt-DNA (data not shown). Therefore, we suggest that the stranded circular DNA, but not long duplex or blunt duplex 69mer and the Y-shaped short DNA region from single-for the 20mer separation. RECQ5/QE separated the 20mer, 69mer from the complementary ssDNA was twice that needed (Fig. 3B). The amount of protein required to separate the DNA fragment separated from the single-stranded circular DNA completion within 10 min. The quantity of the labeled fragment was displaced within 3 min, and the reaction was close to thorough the course of RECQ5/QE-mediated strand displacement is dis-

not Zn²⁺ (Table 1). This specific requirement of divalent cations for activity is similar to that observed with the E. coli RecQ protein (18). Monovalent ions inhibited the ATPase activity (50% inhibition with 65 mM NaCl). The ATPase activity of the purified protein was stimulated by ssDNA, while dsDNA had no effect (Fig. 2).

The RECQ5/QE protein is a DNA helicase
To determine if the ATP hydrolysis by RECQ5/QE is coupled to the helicase activity, we examined whether the purified enzyme could displace a ³²P-labeled oligodeoxynucleotide from single-stranded circular M13mp18 virion DNA or double-stranded circular M13mp18 RFI DNA (open diamonds), and for the GTase in the absence (closed circles) and presence of ssDNA (open circles) or dsDNA (open squares).

![Figure 2. RECQ5/QE is a ssDNA-dependent ATPase and GTase. Reaction mixtures (20 µl) containing purified RECQ5/QE protein and [¹⁴C]ATP or [³²S]GTP were incubated at 27°C, as described in the Materials and Methods. Aliquots of the reaction mixture were taken at various time-points to determine the initial velocity of the reaction. The initial reaction rates are indicated for the ATPase in the absence (closed triangles) and presence (open triangles) of single-stranded circular M13mp18 virion DNA or double-stranded circular M13mp18 RFI DNA (open diamonds), and for the GTase in the absence (closed circles) and presence of ssDNA (open circles) or dsDNA (open squares).](image)

Table 1. ATPase and GTase requirements

<table>
<thead>
<tr>
<th>Condition</th>
<th>ADP (nmol)</th>
<th>GDP (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>14.3</td>
<td>21.2</td>
</tr>
<tr>
<td>-RECQ5/QE</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>-Mg²⁺</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>-Mg²⁺, +Mn²⁺ (2 mM)</td>
<td>11.6</td>
<td>19.7</td>
</tr>
<tr>
<td>-Mg²⁺, +Ca²⁺ (2 mM)</td>
<td>13.4</td>
<td>5.1</td>
</tr>
<tr>
<td>-Mg²⁺, +Zn²⁺ (2 mM)</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>+EDTA (20 mM)</td>
<td>0.0</td>
<td>0.1</td>
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The RECQ5/QE protein (0.4 pmol for ATPase and 0.2 pmol for GTase) was assayed under standard conditions for ATPase and GTase activities, as described in the Materials and Methods.

The RECQ5/QE protein exhibits GTase activity in the presence of ssDNA
In the presence of GTP, the RECQ5/QE protein sustained weak DNA strand separation (Fig. 4). The purified protein was tested for its ability to hydrolyze GTP in the presence and absence of DNA. Unexpectedly, a ssDNA-dependent GTase activity that was several fold higher than the ATPase activity was readily detected (Fig. 2). The GTP hydrolysis required the presence of ssDNA, but was not activated by dsDNA (Fig. 2). The substrate turnover rate of GTase using M13mp18 virion DNA as a co-factor was 75.8 s⁻¹ (3.63 s⁻¹ without DNA). The GTase activity was readily detected (Fig. 2). The GTPase activity was required for strand separation activity. ATP (2 mM) was replaceable by dATP, although other common nucleotide triphosphates either could not, or only weakly supported the DNA strand separation catalyzed by RECQ5/QE. These cofactor requirements are similar to those observed for E.coli RecQ and the small isoform of RECQ5(13,18).

Both ATPase and GTase activities are intrinsic to the RECQ5/QE protein
The ATPase and GTase activity rates of RECQ5/QE were plotted as functions of the ATP and GTP concentrations, respectively (Fig. 5A). The apparent Kₘ values of ATP and GTP were 0.39 and 0.88 mM, respectively. Further characterization of the enzyme revealed that the ATPase activity was inhibited competitively by GTP and its analogs (Fig. 5B). Conversely, the GTase activity was strongly inhibited in a competitive manner by ATP and its analogs (Fig. 5C). UTP and CTP did not inhibit the ATPase and GTase activities as strongly. The optimum reaction conditions, in terms of pH, metal ions and salt, were quite similar for both activities. The purified protein exhibited both the ATPase and GTase activities in the presence of ssDNA, but not dsDNA. Both the ATPase and GTase activities were...
concomitantly inhibited by specific antibodies against RECQ5/QE (Fig. 5D). These data suggest that the active centers for the ATPase and GTPase activities are the same or indistinguishable in the RECQ5/QE protein.

We conclude that both the ATPase and GTPase activities are intrinsic properties of the RECQ5/QE protein, based on the following observations: (i) the purified protein possesses both ATPase and GTPase; (ii) a significant correlation is noted between the ATPase and GTPase activities and the presence of the RECQ5/QE protein throughout the purification steps in multiple preparations, while the corresponding activities were absent from extracts prepared from a mock infection of virus; (iii) GTP and GTP analogs competitively inhibit the ATPase activity; (iv) ATP and ATP analogs competitively inhibit the GTPase activity; (v) both activities display almost identical characteristics in terms of their optimal reaction requirements; (vi) GTP, albeit a weaker binding substrate than ATP, is also used in the DNA helicase activity of RECQ5/QE; and (vii) both the ATPase and GTPase activities were neutralized by anti-RECQ5/QE antibodies.

GTP stimulates the helicase activity of RECQ5/QE

Since ATP and GTP bind to either the same or an indistinguishable site of RECQ5/QE, we explored the possibility that GTP affects the strand separation activity of the protein. GTP resulted in weaker DNA helicase activity than ATP (Fig. 4). The GTPase activity of the RECQ5/QE protein was higher than that of the ATPase (Fig. 2). To date, we have not established a function of RECQ5/QE that specifically requires GTP hydrolysis. GTP supported only weak helicase activity (Fig. 6, lane 7), but did not inhibit the ATP-dependent DNA helicase activity (Fig. 6, lane 6). Surprisingly, stimulation of the ATP-dependent helicase activity by GTP was observed in the presence of a limited amount of RECQ5/QE (Fig. 6, lane 5 versus 6). Under these conditions, ATP strongly inhibited the ssDNA-dependent GTPase activity of the RECQ5/QE protein, while GTP reduced the ssDNA-dependent ATPase activity to approximately half the original value. Since guanosine 5‘-O-(thiotriphosphate) (GTPγS) inhibited the ssDNA-dependent ATPase of RECQ5/QE to the same extent as GTP, a non-hydrolyzable GTP analog, GTPγS, was employed to distinguish whether the stimulation of activity was caused by GTP binding or hydrolysis. We observed that GTPγS stimulated the ATP-dependent DNA helicase activity to a greater extent than GTP (Fig. 6, lane 4). Therefore, we propose that the binding of GTP to RECQ5/QE specifically stimulates its DNA helicase activity.

GTP binding to RECQ5/QE is needed for stimulation of the helicase activity

GTPγS neither supported the helicase reaction itself (Fig. 6, lane 12) nor induced the DNA helicase activity in the presence of GTP (Fig. 6, lane 9). Moreover, ATPγS did not support the
helicase reaction (Fig. 6, lane 11), was inhibitory to this reaction (Fig. 6, lane 3), and did not induce the DNA helicase activity in the presence of GTP (Fig. 6, lane 8). GTPgS was slowly hydrolyzed by the GTPase, while guanosine 5'-[(β,γ-imidotriphosphate) (GMPPNP) was not. GMPPNP activated the DNA helicase activity in the presence of ATP, in contrast to GDP (Fig. 7). We noted that the stimulation was GTP analog-specific and concentration-dependent (data not shown). Furthermore, GTP hydrolysis was not necessary for triggering the activity, although the requirement for GTP binding was evident. Although non-hydrolyzable GTP analogs inhibited the ATPase activity of the RECQ5/QE protein, the remaining activity associated with strand separation was not only sufficient, but was actually enhanced.

Helicase activity stimulation by GTP analogs is caused by an increase in the strand separation rate in the RECQ5/QE helicase reaction

GMPPNP and GTPγS reduced the DNA-dependent ATPase activity of RECQ5/QE to approximately half of that observed under stimulating conditions. In the helicase assay, a labeled 20mer annealed to single-stranded circular M13mp18 DNA (7249b). The overall level of ATP hydrolysis reflected the presence of a large ssDNA region. The stimulatory effects of GMPPNP or GTPγS were specific for the helicase reaction. This stimulation may be caused by the efficient recognition of the substrate, ATP or DNA, or an increase in the strand separation rate during the RECQ5/QE helicase reaction. GMPPNP did not affect the Km value for DNA (0.5 mM) or the S0.5 value for ATP (0.28 mM) in the DNA helicase reaction, but led to an increase in the Vmax value of the DNA helicase reaction (Fig. 8). The addition of GTP and GTP analogs did not affect the polarity of the RECQ5/QE helicase (data not shown). Therefore, GTP binding possibly stimulates the strand separation rate and/or the processivity of the DNA helicase.

DISCUSSION

RECQ5/QE has a unique ssDNA-dependent GTPase activity

In this study, we demonstrated that RECQ5/QE is a DNA-dependent GTPase and ATPase, and an ATP-dependent DNA helicase. Furthermore, GTP binding stimulated the DNA helicase activity. Most helicases favor ATP as their energy source, although in some cases GTP may be utilized in addition to ATP (e.g. HSV-1 helicase-primase (19), E.coli...
DNAB (20) and T4 gp41 (21). The RECQ5/QE protein prefers ATP for the helicase activity, but is more active in hydrolyzing GTP than ATP on ssDNA. No ssDNA-dependent GTPase similar to RECQ5/QE has been reported so far. Helicase motifs share some homology with GTP-binding consensus sequences (22). A comparison of the RECQ5/QE amino acid sequence with other members of the RecQ family and GTPases revealed similarities around the helicase motif I and in the GTPase P-loop [G-1 (23)] (Fig. 9). Interestingly, the *E. coli* FtsZ protein usually behaves as a GTPase. However, a single amino acid substitution (G to S, producing FtsZ84) results in a change in the activity to an ATPase (24). This substitution at the phosphoryl-binding site (25) is associated indirectly with purine recognition (adenine and guanine). Therefore, it is possible that the GTPase and ATPase active centers are the same in the RECQ5/QE protein. This position is well conserved in most RecQ family members (L or V), but varies in *Dm* RECQ5/QE (S), *Ce* E03A3.2 (S), *Hs* RECQ5(C), *Mm* RECQ5(C) and *Dm* RTS (T). It would be interesting to determine whether these RecQ proteins additionally exhibit the GTPase activity.

**RECQ5/QE is an ATP-dependent DNA helicase**

Recently, the small RECQ5 isoform (54 kDa) was purified and characterized (13). The ATPase characteristics of this protein are quite similar to those of our large isoform (120 kDa), and its DNA helicase activity may also be comparable (i.e. low processivity and polarity). Significantly, the DNA unwinding reaction catalyzed by the small RECQ5 isoform requires unexpectedly high protein concentrations. On the other hand, our large isoform, RECQ5/QE, appears to be more active in the helicase reaction. These results are consistent with the results from *in vitro* translation products. Previously, we demonstrated that the full-length (amino acids 1–1058) and the N-terminal half (1–584) of RECQ5/QE showed DNA helicase activity (8). The small RECQ5 isoform (1–473) exhibited very weak DNA helicase activity using *in vitro* translation products (K. Kawasaki, unpublished results).

**RECQ5/QE is an oligomeric helicase**

The native molecular mass of the RECQ5/QE protein was ~480 kDa, and no RECQ5/QE protein was evident in smaller molecular mass fractions (Fig. 1). The DNA helicase family is categorized into at least two classes, specifically, oligomeric ring and monomeric forms (26, 27). Physical data indicate that the RECQ5/QE DNA helicase belongs to the oligomeric type. The BLM helicase, a member of the RecQ family, exists as a hexamer (28). Harvey and Kowalczykowski (29) demonstrated that the *E. coli* RecQ protein has a Hill coefficient of 3.3. The active complex formed upon RecQ helicase binding to DNA substrates is expected to be oligomeric, since multiple ATP-binding sites are utilized by the protein to achieve strand unwinding.
Figure 9. Presence of a GTP-binding motif in the RECQ5/QE protein sequence. (A) Comparison of amino acid sequences around the helicase motif I of RecQ family members. The RecQ helicases are specified on the left. The protein sequence alignment was performed using the Clustal W program (36). Residues indicated with dark or light shades represent identical or similar amino acids, respectively. Numbers on the right represent positions of amino acids from the first methionine. *Dm* RECQ, *D. melanogaster* RECQ5/QE (8); *Hs* RecQ5, *Homo sapiens* RecQ5L (7); *Mm* RECQ5, *Mus musculus* RecQ5L (37); *Ce* E03A3.2, *Caenorhabditis elegans* E03A3.2 (38); *Dm* RTS, *D. melanogaster* RecQ4 (10); *Dm* BLM, *D. melanogaster* BLM (9); *Hs* WRN, *H. sapiens* Wrn (3); *Hs* BLM, *H. sapiens* Bln (1); *Hs* RTS, *H. sapiens* Rts (7); *Hs* RECQLQ, *H. sapiens* ATPaseQ1/RecQ8 (39); *Sp* Rqh1, *Schizosaccharomyces pombe* Rqh1/Rad12 (40); *Sc* SGS1, *Saccharomyces cerevisiae* Sgs1 (41); *Ec* RECQ, *E. coli* RecQ (42). (B) Comparison of the RECQ5/QE helicase motif I and the GTPase P-loop. *Ec* FisZM, *E. coli* FisZ (24); GTPase G-1, consensus sequence motif G-1 present in members of the GTPase superfamily (23). The underlined residue indicates a single amino acid substitution within FisZ84.

separation. These findings collectively suggest that members of the RecQ family are oligomeric DNA helicases. It should be noted that non-hydrolyzable analogs of helicase substrates assemble subunits into stable hexamers [dTTMPPNP for T7 gp4 (30) and ATPγS/GTPγS for T4 gp 41 (21)]. However, Superose 6 gel filtration experiments revealed that ATPγS did not change the apparent molecular mass of RECQ5/QE (K. Kawasaki, unpublished results). It remains to be determined whether ATPγS has the ability to assemble the RECQ5/ QE protein into its active hexameric form in the presence of DNA (30).

Role of ATP hydrolysis in the RECQ5/QE helicase activity

We observed that ATP drove the RECQ5/QE helicase activity, whereas GTP had little effect (Fig. 4). However, the RECQ5/QE protein hydrolyzes GTP on ssDNA more efficiently than ATP (Fig. 2). These data suggest that the mechanism of the ATP-dependent DNA binding/release differs from that of the ATP-dependent DNA binding/release. Since the RECQ5/ QE helicase is oligomeric, it is possible that the protein subunits work cooperatively when utilizing ATP, but not GTP. Consequently, the GTPase activity of RECQ5/QE is not coordinated to DNA binding/release for strand separation. Biochemical analyses of the NS-1 protein of the mouse minute virus and the UL5 protein of Herpes simplex virus revealed that most of the mutants retained significant levels of ATPase activity, while point mutations in the ATP-binding domain severely reduced the helicase activity (31,32). Accordingly, we hypothesize that the ATP-binding domain is important for the coupling of the ATPase and helicase activities. The ATP-binding sites of RECQ5/QE function in both the ATP hydrolysis and the coupling of the ATPase and helicase activities. Although GTP hydrolysis occurs at the same or an indistinguishable site as that for ATP hydrolysis, the former reaction does not accomplish efficient strand separation. The non-hydrolyzable GTP analogs, GTPγS and GMPPNP, also bind RECQ5/QE. Therefore, it is possible that GTP analogs induce a specific conformation in RECQ5/QE through interactions with the ATP-binding site.

Possible mechanisms of helicase stimulation by GTP analogs

Despite the partial inhibition of the RECQ5/QE ATPase activity by GTPγS and GMPPNP, the strand separation activity was not inhibited, but rather, was stimulated. This implies that GTP analogs modulate the DNA helicase activity of RECQ5/QE under conditions where a decreasing supply of energy is available from ATP hydrolysis. Since the $S_{0.5}$ ($K_m$) values for DNA and ATP remained unchanged in the absence and presence of GTP analogs during the RECQ5/QE helicase reaction, the increase in $V_{max}$ may be explained by an elevated strand separation rate and/or processivity. The helicase reaction consists of multiple steps, specifically, oligomerization, ssDNA binding, encounter with the dsDNA region, ATP hydrolysis, DNA binding/release, coordination between ATP hydrolysis and DNA, polar translocation on DNA, displacement from DNA, and recycling. It is therefore important to determine the specific step of the RECQ5/QE reaction that is influenced by GTP analogs. There are several possible explanations for the stimulation by GTP analogs. One hypothesis is that the population of active enzyme is increased upon preventing the formation of a non-productive enzyme–DNA complex in the presence of GTP analogs. The helicase activity of RECQ5/QE is inhibited by ssDNA, presumably because the protein is sequestered by ssDNA. Therefore, the GTP analogs may increase the effective concentration of RECQ5/QE by blocking the sequestration of the enzyme. However, this possibility is unlikely, since the gel mobility shift experiments demonstrated that GTP analogs do not affect the preference or the strength of the RECQ5/QE DNA binding (K. Kawasaki, unpublished results). Another potential explanation is that GTP analogs stimulate the formation of an oligomeric helicase. The RECQ5/QE helicase exhibits an apparent native molecular mass corresponding to a tetrameric protein, while RecQ is a hexameric helicase. However, the native molecular mass of RECQ5/QE remained unchanged on Superose 6 gel filtration in the presence of GTPγS (K. Kawasaki, unpublished results). In addition, a pre-incubation of the RECQ5/QE helicase with GTP analogs did not cause stimulation of the helicase reaction. We cannot exclude the possibility that the active oligomeric formation is too fast to be detected, and is dependent on DNA. A third explanation for the stimulation of the RECQ5/QE helicase by GTP analogs is that these compounds directly enhance the
translocation rate and/or the processivity of the RECQ5/QE helicase during strand separation. Non-catalytic nucleotide binding sites have been found in the F1-ATPase protein, which shares structural similarity with hexameric helicases (33). In this case, it is thought that the non-catalytic sites ensure cooperative catalysis between the catalytic sites. A study by Singleton et al. (34) demonstrated that, in the complex of homo-hexameric T7 gene 4 helicase with ADPNP, only four of the six sites bind nucleotides at any time. GTP analogs may define the non-catalytic sites in the RECQ5/ QE oligomer, which ensures cooperativity between the catalytic sites for efficient strand separation. It is currently unclear how a homooligomeric helicase contains non-catalytic (permanently inactive) or empty, but active sites for NTP/NDP binding (26,35). It remains to be determined whether the binding of GTPγS or GMPPNP similarly defines non-catalytic sites in RECQ5/ QE helicase. Note that the above possibilities for the stimulation of RECQ5/ QE helicase by GTP analogs are not exclusive of each other.

This study is an initial characterization of the large isoform of the RECQ5/ QE helicase, and suggests a new mechanism of regulation or activation of the helicase activity. The RECQ5/ QE protein is accumulated in early embryos (K. Kawasaki, unpublished results; 8). The helicase activity stimulation implies that RECQ5/ QE may be responsible for a quick response to DNA repair or/and processing of stalled replication forks in early embryonic DNA replication. The RECQ5/ QE helicase activity is regulated by cofactors or unknown effectors inducing an active or suppressive state.

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