Analysis of the unwinding activity of the dimeric RECQ1 helicase in the presence of human replication protein A

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ABSTRACT

RecQ helicases are required for the maintenance of genome stability. Characterization of the substrate specificity and identification of the binding partners of the five human RecQ helicases are essential for understanding their function. In the present study, we have developed an efficient baculovirus expression system that allows us to obtain milligram quantities of recombinant RECQ1. Our gel filtration and dynamic light scattering experiments show that RECQ1 has an apparent molecular mass of 158 kDa and a hydrodynamic radius of 5.4 ± 0.6 nm, suggesting that RECQ1 forms dimers in solution. The oligomeric state of RECQ1 remains unchanged upon binding to a single-stranded (ss)DNA fragment of 50 nt. We show that RECQ1 alone is able to unwind short DNA duplexes (<110 bp), whereas considerably longer substrates (501 bp) can be unwound only in the presence of human replication protein A (hRPA). The same experiments with Escherichia coli SSB show that RECQ1 is specifically stimulated by hRPA. However, hRPA does not affect the ssDNA-dependent ATPase activity of RECQ1. In addition, our far western, ELISA and co-immunoprecipitation experiments demonstrate that RECQ1 physically interacts with the 70 kDa subunit of hRPA and that this interaction is not mediated by DNA.

INTRODUCTION

The maintenance of chromosome stability is a key process for the faithful transmission of genetic information as well as for the correct functioning of a cell. The RecQ helicases form an increasingly studied class of enzymes that play a key role in genome preservation (1,2). In humans, three genetic disorders associated with inherent genomic instability arise as a consequence of abnormalities in three different members of the RecQ family of helicases, named BLM, WRN and RECQ4 (3–5). The name RecQ originates from the first helicase of the family discovered in Escherichia coli (6,7). Since this discovery, many other RecQ helicases have been found in different organisms ranging from prokaryotes to eukaryotes (8–11). Five members of the RecQ family have been found in human cells: BLM, RECQ1 (also known as RECQL), RECQ4, RECQ5 and WRN (2,12). These five helicases are characterized by N- and C-terminal tails of different lengths, whose function still demands further investigation, and by a conserved central domain of ~450 amino acids. This domain contains the seven motifs responsible for the unwinding activity of the RecQ helicases, also present in helicases from other families (2,13). The C-terminal tails of BLM, WRN, RECQ5β and RECQ1 contain a signature motif referred to as the RecQ-Conserved (RQC) domain that is unique to RecQ helicases and most likely mediates the interaction with other proteins (14,15). The BLM and WRN helicases contain an additional conserved motif in their C-terminal tail called the helicase and RNase-D-C-terminal (HRDC) domain that is involved in single-stranded (ss)DNA binding (14,16). Only the helicase activities of BLM, WRN, RECQ1, a Drosophila variant of RECQ5, Saccharomyces cerevisiae Sgs1 and E.coli RecQ have been carefully characterized (17–23). From these kinetic studies, it is evident that although they all unwind DNA with a 3’→5’ polarity, their substrate specificity is not identical (21). In addition, the in vivo activity and function of the RecQ helicases is likely to be controlled by other proteins that directly interact with them, such as replication protein A (24,25), Ku heterodimer (26,27), DNA polymerase δ (28), p53 (29–31), DNA polymerase β (32), TRF2 (33), proliferating cell nuclear antigen (PCNA) (34) and RAD52 (35). Some of these proteins interact with the N- and C-terminal tails of the helicases, while others bind regions of the central domain. For example, recent studies have shown that the 70 kDa subunit of the Ku heterodimer interacts with the N-terminal tail of WRN, while the 80 kDa subunit interacts with the C-terminus (27). The N-terminal domain of WRN is also involved in its interaction with PCNA (34), while the C-terminal region mediates its binding to p53 (31,36) and to FEN1 through the RQC domain (37). Similarly, the extended N- and C-terminal

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tails of BLM helicase are responsible for its interaction with topoisomerase III (38), MLH1 (39,40) and RAD51 (41). A thorough search of proteins that interact with and affect the activity of the human RecQ helicases is of fundamental importance to understanding their mechanism of DNA unwinding in vivo and for a better comprehension of their function.

Recently, the crystal structure of the catalytic core of the E.coli RecQ helicase has been solved in its DNA unbound form (42). The molecule forms a Y-shaped structure with a major cleft on its surface and is composed of four subdomains. The two N-terminal subdomains form the helicase region, while the remaining two form the RQC domain. Interestingly, one of the two RQC subdomains forms a layer of helices that binds Zn\(^{2+}\) through four conserved cysteine residues, while the other forms a so called winged helix (WH) subdomain that shares significant structural similarities with other DNA-binding proteins. The structure of E.coli RecQ is more closely related to helicases of the SF2 family, such as the hepatitis C virus NS3 helicase (43), than to SF1 members such as PcrA (44). Nevertheless, the mode of ATP binding seems to be quite similar to that observed for PcrA and a conserved patch of aromatic and charged residues used in PcrA for ssDNA binding is also present in E.coli RecQ, suggesting that these enzymes may use a similar mechanism to bind and unwind DNA. Resolution of the crystal structure provided key indications on the mechanism that E.coli RecQ may adopt to unwind DNA. Nonetheless, several aspects still demand further investigation. For example, although the structure shows that the core domain of E.coli RecQ is monomeric, there is conflicting evidence on the oligomerization state of the full-length protein (22). Kinetic studies indicated that E.coli RecQ forms a multimer of at least three subunits in solution (22). In contrast, gel filtration experiments suggested that E.coli RecQ runs as a monomer (45) and recent biophysical studies provided evidence that this enzyme is also in its monomeric form when bound to single-stranded chains and when unwinding DNA (23). Thus, further studies with E.coli RecQ as well as with the other members of the RecQ helicase family will be crucial to learn whether the RecQ helicases share common structures and mechanisms of DNA unwinding.

In the present work, we have expressed RecQ1 in baculovirus, we have determined its oligomerization state and we have investigated its catalytic activity with DNA substrates of increasing length. We have previously observed that the helicase activity of RecQ1 purified from HeLa cells is more closely related to helicases of the SF2 family, such as the hepatitis C virus NS3 helicase (43), than to SF1 members such as PcrA (44). Nevertheless, the mode of ATP binding seems to be quite similar to that observed for PcrA and a conserved patch of aromatic and charged residues used in PcrA for ssDNA binding is also present in E.coli RecQ, suggesting that these enzymes may use a similar mechanism to bind and unwind DNA. Resolution of the crystal structure provided key indications on the mechanism that E.coli RecQ may adopt to unwind DNA. Nonetheless, several aspects still demand further investigation. For example, although the structure shows that the core domain of E.coli RecQ is monomeric, there is conflicting evidence on the oligomerization state of the full-length protein (22). Kinetic studies indicated that E.coli RecQ forms a multimer of at least three subunits in solution (22). In contrast, gel filtration experiments suggested that E.coli RecQ runs as a monomer (45) and recent biophysical studies provided evidence that this enzyme is also in its monomeric form when bound to single-stranded chains and when unwinding DNA (23). Thus, further studies with E.coli RecQ as well as with the other members of the RecQ helicase family will be crucial to learn whether the RecQ helicases share common structures and mechanisms of DNA unwinding.

In the present work, we have expressed RecQ1 in baculovirus, we have determined its oligomerization state and we have investigated its catalytic activity with DNA substrates of increasing length. We have previously observed that the helicase activity of RecQ1 purified from HeLa cells is specifically stimulated by human replication protein A (hRPA), similarly to BLM and WRN (20). In this study, we have carried out far western, ELISA and co-immunoprecipitation experiments to demonstrate that RecQ1 physically interacts with hRPA and that nucleic acids do not mediate this interaction. The contribution of hRPA to the ATPase and helicase activities of RecQ1 has been carefully investigated.

**MATERIALS AND METHODS**

**Reagents**

All salts, bovine serum albumin (BSA), dithiothreitol (DTT) and phenylmethylsulfonyl fluoride were from Sigma (St Louis, MO, USA). The M13mp18 single-stranded (ss)DNA plasmid, T4 DNA ligase and XbaI, NheI and XhoI restriction enzymes were from New England BioLabs. The SuperScript™ II RNase H reverse transcriptase, Platinum® Pfx DNA Polymerase, pFastBac1 donor plasmid, kanamycin, gentamicin, tetracycline, Blue-gal, IPTG, Sf-900 II SFM medium and fetal bovine serum were from Invitrogen. The protease inhibitor cocktail was from Roche Molecular Biochemicals. All reagents were from Sigma (St Louis, MO, USA). The radioactive nucleoside triphosphates were obtained from Amersham Biosciences (Little Chalfont, UK). The T4 polynucleotide kinase and sequencing grade porcine trypsin for protein digestion were from Promega (Madison, WI, USA). Recombinant hRPA containing all three subunits (RPA70, RPA32 and RPA14) was expressed in and purified from E.coli according to the previously described protocol (46).

**Expression and purification of recombinant RecQ1**

The human RecQ1 cDNA encoding 649 amino acids was amplified from lymphocytes prepared from human peripheral blood using reverse transcription (RT) PCR and was cloned into the Xhel and XhoI restriction sites of the pET-28a(+) vector (Novagen). The sequence of RecQ1 cDNA was verified by DNA sequencing. A 2.1 kb DNA fragment, containing the coding sequences for six histidines, a thrombin cleavage site and the full-length human RECQ1, was obtained by XbaI and XhoI digestion. This DNA fragment was then inserted into the pFastBac1 vector. Recombinant bacmids were prepared according to the manufacturer’s instructions using the pFastBac1 transfer vector (Invitrogen). SF9 cells were transfectected with the recombinant bacmid DNA. In this way, the recombinant baculovirus expressing histidine-tagged RecQ1 protein was produced. The amino acid sequence preceding the 649 amino acids of RecQ1 protein is MGSSHHHHHHSSGLVPRGSHMAS, containing six histidines, a linker region and a thrombin cleavage site. The baculovirus was used to infect SF9 cells cultured in suspension (suspension culture ~10^6 cells/ml). Seventy-two hours after infection, cells were harvested by centrifugation and were resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 400 mM NaCl, 5 mM β-mercaptoethanol, 1% Nonidet P-40) supplemented with protease inhibitor cocktail (Roche Molecular Biochemicals). An aliquot of 12 ml of lysis buffer was used for 150 ml of suspension culture (1.5 × 10^6 cells) and incubation was for 15 min at 4°C with gentle agitation. Recombinant RecQ1 was identified in the cell lysate by SDS–PAGE. The lysate was cleared by centrifugation (15 000 r.p.m. at 4°C, Sorvall SA600 rotor) and then incubated with TALON metal affinity resin (Clontech) (1 ml resin/5 mg protein) for 2 h at 4°C. The resin was washed with buffer (20 mM Tris–HCl, pH 8.0, 5 mM β-mercaptoethanol, 12.5 mM imidazole) containing 500 mM NaCl (two washes) and 100 mM NaCl (two washes). The polyhistidine-tagged RecQ1 was eluted in buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5 mM β-mercaptoethanol, 120 mM imidazole). The purity of the preparation was verified by SDS–PAGE with silver staining. The identity of the purified product was verified by both mass
spectrometry, sequencing and immunoblotting with a specific monoclonal antibody against the hexa-histidine tag. The recombinant RECQ1 was concentrated and stored in buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM DTT) at –80°C. The concentration of RECQ1 was determined by UV absorption measurements using an extinction coefficient of 280 nm of 67 790 M⁻¹cm⁻¹ estimated from the amino acid sequence (ProtParam, available at www.expasy.org). Approximately 0.75 mg RECQ1 were obtained from 1.5 × 10⁹ infected Sf9 cells.

**Gel filtration experiments**

The TSK-GEL G3000SWXL column (30 cm × 7.8 mm) was equilibrated at a flow rate of 1 ml/min with 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM DTT. The column was then calibrated using gel filtration molecular mass markers containing thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (45 kDa), myoglobin (17 kDa) and vitamin B₁₂ (1.35 kDa). BSA (66 kDa) was also used as a standard. A solution of 2.5 μM RECQ1 (37.5 μg, 200 μl) was loaded onto the column.

**Dynamic light scattering (DLS)**

DLS measurements were performed using a DynaPro-MS/X instrument (Protein Solution, Charlottesville, VA) with a 12 μl quartz cuvette and the scattered light was collected at an angle of 90°. The time-dependent auto-correlation function (ACF) of the photon current was acquired with a built-in software correlator (based on a DSP unit). Samples were gently injected into the cell through a Whatman filter with 0.02 μm porosity. The protein concentration was in the range 2.0–3.0 μM and the buffer used for measurements was 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM DTT (5 mM MgCl₂ and 5 mM ATP were also added in separate experiments). The 50mer ssDNA substrate was added to RECQ1 solutions in slight excess over the protein concentration ([DNA]/[RECQ1] = 1.1–3.0) and the solution was incubated for 30 min at room temperature before collecting data. ACF were incrementally stored every 10 s at a temperature of 23 ± 0.1°C. ACF were analyzed with the Dynamics v6.0 software provided, which contains proprietary algorithms to filter out unavoidable (since the small cuvette volume remains open to the air during measurements) dust contamination of the ACF at longer times.

**EMSA assay**

The recombinant RECQ1 (1 μM) was incubated with 5‘-[γ-³²P]-labeled ssDNA of 50 T (0.4 nm) in a 20 μl reaction mixture containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl and 1 mM DTT. Incubation was for 20 min at room temperature. The resulting mixture was then resolved by 6% non-denaturing PAGE and visualized by autoradiography. Gels were run at 100 V and 4°C in TBE buffer. Labeled DNA fragments were detected in the dried gel by autoradiography (Instant Imager; Packard Instrument Co.).

**ATPase assay**

Standard ATPase assay reaction mixtures (50 μl) contained 20 mM Tris–HCl, pH 7.5, 8 mM DTT, 5 mM MgCl₂, 5 mM ATP, 10 mM KCl, 4% (w/v) sucrose, 80 μg/ml BSA and ³²P-labeled helicase substrate (0.4 nM). The recombinant RECQ1 was added to the mixture and incubated at 37°C for the times specified in the figure legends. The reaction was terminated by the addition of 0.3% SDS, 10 mM EDTA, 5% glycerol and 0.1% bromphenol blue. The products of the reaction were fractionated by electrophoresis on a 12% or 6% non-denaturing polyacrylamide gel. The gel was dried and the extent of DNA unwinding was quantitated by electronic autoradiography (Instant Imager; Packard Instrument Co.).

**DNA helicase assay**

The helicase assay measures the unwinding of a ³²P-labeled DNA fragment from a partial duplex DNA molecule. The 20 μl reaction mixture contained 20 mM Tris–HCl, pH 7.5, 8 mM DTT, 5 mM MgCl₂, 5 mM ATP, 10 mM KCl, 4% (w/v) sucrose, 80 μg/ml BSA and ³²P-labeled helicase substrate (0.4 nM). The recombinant RECQ1 was added to the mixture and incubated at 37°C for the times specified in the figure legends. The reaction was terminated by the addition of 0.3% SDS, 10 mM EDTA, 5% glycerol and 0.1% bromphenol blue. The products of the reaction were fractionated by electrophoresis on a 12% or 6% non-denaturing polyacrylamide gel. The gel was dried and the extent of DNA unwinding was quantitated by electronic autoradiography (Instant Imager; Packard Instrument Co.).

**Preparation of DNA helicase substrates**

The DNA substrates, consisting of different ³²P-labeled polynucleotides annealed to M13mp18 phage ssDNA, were constructed as described previously (20). The sequences of all the polynucleotides are complementary to nucleotides of M13mp18 phage ssDNA. The substrates with double-stranded regions of 25 and 50 bp were made using polynucleotides 5‘-ctctagaggatccccgggtaccgag-3‘ (25 bp) and 5‘-gcatctcgctgactagatctgacgccgggtaccgagctcgaatt-3‘ (50 bp), complementary to nucleotides 6239–6263 and 6231–6280 of M13mp18 phage ssDNA, respectively. The substrates with double-stranded regions of 110, 216, 301, 416, 501, 603 and 807 bp were made by PCR amplification of a M13mp18 fragment of the proper length. The forward primer for the PCR was annealed to region 28–47 of M13mp18, whereas the reverse primers were annealed to regions 28–137 for 110 bp, 28–243 for 216 bp, 28–328 for 301 bp, 28–443 for 416 bp, 28–528 for 501 bp, 28–630 for 603 and 28–834 for 807 bp.

**Far western assay**

The far western assay was conducted essentially as described by Brosh et al. (25). Briefly, 0.36 μg recombinant RECQ1, 20 μg BSA, 1.5 μg hRPA and 1.6 μg Ku were loaded onto a SDS–PAGE gel, electrophoresed and transferred to Hybond-P PVDF membrane (Amersham Pharmacia Biotech). All subsequent steps were performed at 4°C. The membrane was immersed twice in denaturation buffer [6 M guanidine HCl in phosphate-buffered saline (PBS)] for 10 min followed by six times for 10 min in serial dilutions (1:1) of denaturation buffer supplemented with 1 mM DTT. The membrane was blocked in PBS containing 5% powdered milk, 0.3% Tween 20 for 30 min before being incubated overnight with RECQ1 (0.8 μg/ml) in PBS supplemented with 0.25% powdered milk, 0.3% Tween 20, 1 mM DTT. The membrane was washed four times for 10 min in PBS containing 0.3% Tween-20, 0.25% powdered milk. The second wash contained 0.0001% glutaraldehyde.
Western analysis was then performed to detect the presence of RECQ1 using an anti-hexa-histidine monoclonal antibody (Clontech) as primary antibody at 1:5000 dilution. Anti-mouse IgG–horseradish peroxidase conjugate (Sigma) was used as the secondary antibody at a 1:15 000 dilution and detected using ECL (Amersham Pharmacia Biotech) following the manufacturer’s instructions. Similar results were obtained without using glutaraldehyde in the second wash. The only difference is that the intensity of the signal was slightly weaker.

ELISA assay

Purified recombinant RECQ1 was diluted to a concentration of 18 mM in carbonate buffer (0.016 M Na₂CO₃, 0.034 M NaHCO₃, pH 9.6) and was added to appropriate wells of a 96-well microtitrter plate (50 μl/well), which was incubated at 4°C. BSA was used in the coating step for control reactions. The samples were aspirated and the wells were blocked for 2 h at 30°C with blocking buffer (PBS, 0.5% Tween 20 and 3% BSA). The procedure was repeated. hRPA was diluted to 144 nM in blocking buffer and was added to the appropriate wells of the ELISA plate (50 μl/well), which was incubated for 1 h at 30°C. For ethidium bromide (EtBr) treatment, 50 μg/ml EtBr was included in the incubation with hRPA during the binding step in the corresponding wells. The samples were aspirated and the wells were washed five times before addition of anti-hRPA (Ab-1) mouse monoclonal antibody (Oncogene Research Products), diluted 1:100 in blocking buffer, and incubation at 30°C for 1 h. Following three washes, horseradish peroxidase-conjugated anti-mouse secondary antibody (1:2500) was added to the wells and the samples were incubated for 30 min at 30°C. After washing five times, any hRPA bound to the RECQ1 was detected using OPD substrate (Sigma). The reaction was terminated after 5 min with 3 N H₂SO₄ and absorbance readings were taken at 490 nm. Data analysis for determination of the apparent dissociation constant (K_d) was performed as previously described (25).

Anti-RECQ1 antibody production

Custom polyclonal anti-RECQ1 antibody was raised against a 20 amino acid peptide corresponding to residues 644–662 (GNFKKAAAANMLQGSKNT) in the C-terminal region of RECQ1 with an N-terminal cysteine conjugated to Sepharose 4B for affinity purification (Alpha Diagnostic International, San Antonio, TX). The peptide was conjugated to KLH carrier protein and raised in rabbit. An IgG fraction from the antiserum was purified using the peptide coupled to Sepharose 4B via a cysteine group. Affinity-purified antibody was checked by ELISA using free peptide.

Co-immunoprecipitation experiments

Nuclear extracts were prepared from exponentially growing HeLa cells as described previously (37). For co-immunoprecipitation experiments, nuclear extract (1.36 mg protein) was incubated with rabbit polyclonal anti-RECQ1 antibody (1:100) in buffer D (50 mM HEPES, pH 7.5, 100 mM KCl, 10% glycerol) for 4 h at 4°C. The mixture was subsequently tumbled with 60 μl of protein G–agarose (Roche Molecular Biochemicals) at 4°C overnight. The beads were then washed three times with buffer D supplemented with 0.1% Tween-20. Proteins were eluted by boiling in SDS sample buffer and the eluate was resolved on 10% polyacrylamide Tris–glycine SDS gels and transferred to PVDF membranes (Amersham Biosciences). The membranes were blocked with 5% non-fat dry milk in PBS containing 0.1% Tween-20 and probed for hRPA and RECQ1 using anti-RECQ1 (1:20) rabbit polyclonal antibody and anti-RPA (Ab-1) mouse monoclonal antibody (1:20) (Oncogene Research Products), respectively, followed by detection with donkey anti-rabbit IgG (Santa Cruz Biotech) or horse anti-mouse secondary (Vector Laboratories Inc.) antibodies conjugated to horseradish peroxidase. RECQ1 and hRPA on immunoblots were detected using ECL Plus (Amersham Biosciences).

Microsequence analysis

The Coomassie blue stained band containing RECQ1 was cut out and digested with sequencing grade bovine trypsin (Promega). The digestion products were separated by micro-high pressure liquid chromatography and analyzed by electrospray ionization mass spectrometry (Finnigan LC DECA; Thermo-Finnigan Corp., San Jose, CA).

RESULTS

Two closely related isoforms of RECQ1 were first cloned independently by two separate laboratories, one encoding a protein of 649 amino acids and the other of 659 amino acids (48,49). The only difference between these two isoforms is found at the C-terminal end. Sequence alignment of the two isoforms indicates that the 659 amino acid form seems to have three additional amino acid substitutions if compared to the shorter variant: A175D, C543S and T566A. Nonetheless, these substitutions may be attributed to sequencing errors since Cys543 especially is highly conserved in the RecQ helicase family. More recently, two other smaller isoforms have been identified, but their enzymatic activities have not been determined (50). In this work, we have PCR amplified the cDNA encoding the 649 amino acid variant of RECQ1 starting from lymphocytes prepared from human peripheral blood and cloned the amplified gene in the appropriate expression vector. The recombinant RECQ1 helicase was expressed in baculovirus at high yield (~0.75 mg RECQ1 from 1.5 × 10⁸ infected SF9 cells) and the purity of the purified protein was tested by SDS–PAGE. The Coomassie stained gel showed only a single band with a molecular mass of ~75 kDa (Fig. 1). The identity of the protein was confirmed by mass spectrometry (Table 1). The activity of recombinant RECQ1 was identical to that of the native RECQ1 purified from a nuclear extract of HeLa cells (20; data not shown).

Analysis of the RECQ1 oligomerization state

We performed gel filtration and DLS experiments in order to investigate the oligomerization state of recombinant RECQ1 in solution (Fig. 2). Gel filtration experiments showed that RECQ1 has an apparent molecular mass of 158 kDa, suggesting that this protein is a dimer in solution (Fig. 2A). This result agrees perfectly with our previous glycerol gradient and gel filtration studies performed with the RECQ1 protein purified from HeLa cells, in which we had measured a sedimentation coefficient of 7.3 ± 1.7 S and a Stokes radius of 49.5 ± 10.5 Å, corresponding to a native
molecular mass of 160 ± 18 kDa (20). To further support these results, DLS measurements have been conducted on RECQ1 in the 2.0–3.0 μM concentration range. The data clearly show that the correlation time of RECQ1 is higher than that measured for BSA (Fig. 2B). This result provides strong experimental evidence that purified recombinant RECQ1 is not a monomer in solution. From an analysis of the data with the Dynamic v6.0 software, we derived a hydration radius of 0.6 nm for RECQ1, compared to a hydration radius of 0.4 nm for the BSA control, which has a molecular mass of 66 kDa. The value of the hydrodynamic radius did not change in the presence of ssDNA fragments of 50 nt, with or without 5 mM MgCl₂ and 5 mM ATP. Using fluorescence we measured a dissociation constant K_d < 0.1 μM for RECQ1 binding to the 50 nt ssDNA fragment, indicating that RECQ1 is fully bound to ssDNA under the reaction conditions used for the DLS experiments (data not shown). Moreover, the complex between RECQ1 and the ssDNA probe is clearly visible in EMsA experiments performed under the same buffer conditions (Fig. 2). Our DLS data indicate that only one RECQ1 complex is able to bind a 50 nt fragment and that RECQ1 maintains the same dimeric structure in the free and DNA-bound forms.

**RECQ1 unwinding of DNA substrates with increasing duplex length**

A series of M13 partial duplex DNA substrates of 17, 25, 50, 110 and 216 bp were prepared in order to characterize the effect of duplex length on the helicase activity of RECQ1. Unwinding experiments were carried out at increasing concentrations of RECQ1 in a buffer containing 20 mM Tris–HCl, pH 7.5, 8 mM DTT, 5 mM MgCl₂, 5 mM ATP, 10 mM KCl, 4% (w/v) sucrose, 80 μg/ml BSA (Fig. 3). The figure shows that while a short DNA duplex of 17 bp can be easily unwound using 1 nM RECQ1, longer DNA duplexes require significantly more enzyme to be opened. The 25 bp duplex was completely unwound in 30 min in the presence of 150 nM RECQ1, while using the same enzyme concentration only 50% of the 50 bp duplex was unwound. A further increase in the concentration of RECQ1 did not result in a significant enhancement of the percent of substrate unwound. The reason why this longer duplex cannot be completely unwound even at high concentrations of RECQ1 may be due to strand reannealing during the unwinding reaction, as already observed for other helicases (25,51). The 110 bp duplex is the longest DNA substrate among those tested that can be partially unwound by RECQ1. In fact, almost 20% of this substrate was unwound in the presence of 250 nM RECQ1 in 30 min, while no unwinding was detected with the 216 bp substrate.

For a better comparison of the results obtained with the different substrates, it is helpful to express the unwinding data as an apparent rate of base pairs unwound per min per RECQ1 helicase unit (bp/min/RECQ1) (25). The apparent unwinding rates for the 25 and 50 bp partial duplexes were almost identical. In fact, at a RECQ1 concentration of 50 nM, the rates were 0.00238 and 0.00240 bp/min/RECQ1, respectively, for the 25 and the 50 bp duplexes. Likewise, at a RECQ1 concentration of 100 nM, the unwinding rate was 0.00234 bp/min/RECQ1 for the 25 bp duplex and 0.00232 for the 50 bp duplex. Thus, the concentration of RECQ1 that is required for unwinding is proportional to the length of the duplex to be unwound.

On the other hand, the unwinding activity of RECQ1 was dramatically reduced with longer DNA duplexes. In fact, only a small fraction of the 110 bp duplex could be unwound by RECQ1 and no unwinding was detected for the 216 bp duplex, even at the highest RECQ1 concentration tested. Therefore, RECQ1 is able to efficiently unwind only short DNA substrates in vitro.

**Stimulation of RECQ1 helicase activity by hRPA**

In our previous study, we showed that the helicase activity of RECQ1 purified from HeLa nuclear extract was specifically stimulated by hRPA (20). On the other hand, the small amount of HeLa RECQ1 available did not allow us to perform studies with DNA duplexes longer than 110 bp. In the present work, recombinant RECQ1 was incubated with the 216 bp duplex

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**Table 1. Amino acid sequences of the peptides of RECQ1 identified by mass spectrometry**

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<tr>
<th>Positions</th>
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<td>QQELIQKK</td>
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<td>71–78</td>
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<td>118–119</td>
<td>EVFLVMPTGGGK</td>
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<td>151–167</td>
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substrate in the presence of increasing concentrations of hRPA (Fig. 4A). In control reactions, we verified that hRPA alone (300 nM) did not denature the 216 bp DNA duplex. Similarly, RECQ1 alone (300 nM) was unable to unwind this substrate, even though more than 16% of the substrate was unwound when hRPA was added at a concentration of 100 nM and 70% unwinding was reached by increasing the hRPA concentration to 300 nM. Moreover, kinetic studies performed in the presence of 300 nM RECQ1 and 300 nM hRPA showed that the 216 bp substrate could be completely unwound if the incubation time was >80 min (Fig. 4B). On the other hand, no unwinding was detected when *E. coli* single-strand binding protein (ESSB) was used instead of hRPA, even at a concentration of 3000 nM.

Strand displacement can be expressed as a function of the ratio (R) of the concentration of SSB units per concentration of DNA-binding site (given by the concentration of the ssDNA substrate in nucleotides divided by the number of oligonucleotides covered by each unit). As such, the analysis takes into account that hRPA covers ~30 nt when binding to DNA (52), while ESSB binds ~35 nt (53). This analysis indicated that 16% of the substrate was unwound at a concentration of hRPA that coated the ssDNA molecule in the helicase reaction (96 nM) and that at an R value of 3, 70% of the substrate was unwound in 45 min. On the other hand, ESSB failed to catalyze unwinding even at a concentration 10-fold higher than that used for hRPA and at an R value of 36.

In order to determine the maximum number of base pairs that can be unwound by RECQ1 in the presence of hRPA, we performed a systematic study using DNA substrates with DNA duplex regions of 301, 416, 501, 603 and 807 bp (Fig. 5). The results show that, under our experimental conditions, RECQ1 is able to unwind up to 501 bp, since no unwinding was detectable for the 603 and 807 bp duplexes.

**Effect of hRPA on the rate of ATP hydrolysis**

The ATPase activity of RECQ1 was measured in the absence and presence of DNA cofactors of varying length. The consequences of the addition of hRPA on the rate of ATP hydrolysis were analyzed (Fig. 6). Little or no ATPase activity was detected in the absence of DNA, in agreement with previous studies performed with other DNA helicases (25,54). In the presence of ssDNA cofactors, ATP hydrolysis followed classical Michaelis–Menten kinetics over an ATP concentration range of 1–800 μM. The kinetic constant ($k_{cat}$) for ATP hydrolysis with a short ssDNA fragment of 17 nt was 31 ± 2 min$^{-1}$. The $k_{cat}$ value increased, by <2-fold in the presence of

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**Figure 2.** Gel filtration and dynamic light scattering analysis. (A) Gel filtration experiments were performed as described in Materials and Methods. The TSK-GEL G3000SWXL column was calibrated with thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (45 kDa), myoglobin (17 kDa) and vitamin B$_{12}$ (1.35 kDa). The amount of RECQ1 loaded was 37.5 μg. The solid curve shows the elution profile of RECQ1 and the dashed curve the elution profile of BSA (66 kDa). (B) Normalized autocorrelation function (ACF) for control BSA (solid circles), RECQ1 alone (open circles) and RECQ1 + 50mer ssDNA + ATP + Mg$^{2+}$ (open squares). Measurements were carried out at room temperature (23 ± 0.1°C) with a protein concentration of ~2.5 μM in buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM DTT). Solid lines are the fit with the built-in regularization method (Dynamics v6.0 from Protein Solutions). Radii were 3.5 ± 0.4 nm for BSA and 5.4 ± 0.6 nm for all the RECQ1 samples. (Insert) Band shift assay with the 50 nt ssDNA probe in the absence (1) and presence (2) of RECQ1. The experiments were carried out with 1 μM RECQ1 and 0.4 nM DNA as described in Materials and Methods.

**Figure 3.** Unwinding studies with DNA substrates of increasing duplex length. Helicase assays were performed as described in Materials and Methods. The indicated amount of RECQ1 was used to unwind the M13mp18 partial duplex substrates of 17 (black diamond), 25 (open square), 50 (black circle), 110 (black triangle) and 216 bp (open triangle). The concentration of the substrate was always 0.4 nM. After incubation at 37°C for 30 min, the reaction mixtures were resolved on a 12% non-denaturing polyacrylamide gel.
a DNA cofactor of 25 nt and by a factor of 4 in the presence of the long M13 ssDNA circle (Table 2). The same experiments were repeated at two different ssDNA concentrations and no change in the initial rate for ATP hydrolysis was observed, indicating that the reaction was saturated with respect to ssDNA (data not shown). The addition of different concentrations of hRPA (from 50 to 300 nM) did not induce an enhancement in \( k_{\text{cat}} \) (Table 2). On the contrary, at the highest hRPA concentration (300 nM) we observed a 2-fold inhibition of the rate of ATP hydrolysis (data not shown). This result is similar to that obtained by Brosh and co-workers studying the effect of hRPA on the ATPase activity of BLM and cannot be easily explained, since it is only observed with M13 and not with the shorter ssDNA effectors (25). Our results indicate that hRPA does not increase the ATPase activity of RECQ1 and that the stimulation of the helicase activity of RECQ1 by hRPA must be due to other factors.

**Far western and ELISA analysis of the RECQ1–hRPA interaction**

We performed far western and ELISA experiments to study the possible physical interaction of RECQ1 with hRPA. For the far western experiments, hRPA was immobilized on a Hybond-P membrane that was subsequently incubated with recombinant RECQ1. After washing the unbound proteins, conventional western analysis was performed to detect the RECQ1 bound to hRPA (Fig. 7A). BSA was added as a negative control. In addition, a membrane containing hRPA was incubated with buffer alone to verify that there was no cross-reactivity of the anti-histidine antibody with hRPA. The far western analysis showed a single band at the position of the 70 kDa subunit of hRPA, while no binding was detected for BSA and for the 32 and 14 kDa subunits of hRPA. This result indicates that RECQ1 specifically interacts with the 70 kDa subunit of hRPA.
subunit of hRPA. The same experiment carried out with the Ku heterodimer showed that RECQ1 does not interact with Ku.

To further confirm a direct association between RECQ1 and hRPA, we performed ELISA experiments with the recombinant purified proteins. Increasing amounts of hRPA were incubated in wells that had been previously coated with RECQ1 (18 nM) (Fig. 7B). After washing, the RECQ1–hRPA complex was detected with a mouse monoclonal antibody raised against the 70 kDa subunit of hRPA and a colorimetric assay was used to build binding curves that reached saturation at hRPA concentrations >40 nM. The specificity of this interaction was demonstrated by the absence of color in wells that had been precoated with BSA rather than RECQ1. Data analysis yielded an apparent dissociation constant ($K_d$) of 6.2 nM, which is similar to that previously described for the interaction of BLM with hRPA (25). Moreover, the same experiments performed in the presence of EtBr (50 µg/ml) yielded almost identical binding curves demonstrating that the RECQ1–hRPA interaction is not mediated by DNA (data not shown).

Figure 5. Unwinding studies with DNA substrates of increasing duplex length in the presence of hRPA. The indicated amount of hRPA was preincubated with different DNA substrates containing partial duplexes of 301 (filled diamond), 416 (open square), 501 (filled circle), 603 (open triangle) and 807 bp (filled square). The concentration of the substrate was always 0.4 nM. The reactions were initiated by adding 300 nM RECQ1. The reaction was incubated at 37°C for 120 min. The reaction mixtures were resolved on a 6% non-denaturing polyacrylamide gel. The percentage of unwinding is expressed as a function of hRPA concentration. The hRPA concentrations (nM) are indicated above each lane in the autoradiogram. Lanes C and D are control assays without enzyme and with heat-denatured substrate.

Figure 6. Analysis of the ATPase activity of the recombinant RECQ1 and effect of hRPA on rate of ATP hydrolysis. Filled circles, reactions with M13mp18; open circles, reactions with M13mp18 and hRPA (50 nM); filled squares, reactions with 25 nt ssDNA; open squares, reactions with 25 bp ssDNA and hRPA (50 nM); filled triangles, reactions with 17 bp ssDNA. The initial velocities for ATP hydrolysis were expressed as a function of the ATP concentration. The experimental points were fitted to the Michaelis–Menten equation: $V_0 = V_{max}X/(K_m + X)$, where $V_0$ is the initial velocity and $X$ is the substrate concentration (ATP). Each value represents the mean of at least five independent measurements.

Table 2. Comparison of the $k_{cat}$ (min$^{-1}$) for ATP hydrolysis of RECQ1, BLM and WRN

<table>
<thead>
<tr>
<th></th>
<th>17mer</th>
<th>25mer</th>
<th>M13mp18</th>
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<tbody>
<tr>
<td>hsRECQ1</td>
<td>31.14 ± 2.29</td>
<td>56.95 ± 1.51</td>
<td>126.29 ± 3.44</td>
</tr>
<tr>
<td>hsRECQ1 + hRPA</td>
<td>58 ± 1.48</td>
<td>133.18 ± 3.44</td>
<td></td>
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<tr>
<td>hsBLM</td>
<td>1163 ± 358$^a$</td>
<td>1407 ± 72$^b$</td>
<td></td>
</tr>
<tr>
<td>hsBLM + hRPA</td>
<td>1328.4$^c$</td>
<td>58.4 ± 17$^c$</td>
<td></td>
</tr>
<tr>
<td>hsWRN</td>
<td>69.7 ± 20$^c$</td>
<td>900 ± 120$^d$</td>
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</table>

The $k_{cat}$ values for RECQ1 were measured in the presence of various DNA effectors with and without hRPA.

$^a$Brosh et al. (25).

$^b$Janscak et al. (61).

$^c$Brosh et al. (24).

$^d$Ozsoy et al. (11).
Co-immunoprecipitation experiments

We performed co-immunoprecipitation experiments from HeLa nuclear extracts using a polyclonal antibody directed against RECQ1 to make sure that a physical interaction between RECQ1 and hRPA also exists in cell nuclei. The anti-RECQ1 antibody precipitated both RECQ1 (data not shown) and hRPA proteins from normal human nuclear extract (Fig. 8, lane 4). Approximately 5% of the hRPA from the HeLa nuclear extract input (Fig. 8, lane 2) was co-immunoprecipitated with RECQ1 using the anti-RECQ1 antibody. Control experiments using normal rabbit IgG incubated in the HeLa nuclear extracts confirmed the specificity of the anti-RECQ1 antibody (Fig. 8, lane 5). Analogous experiments carried out in the presence of EtBr demonstrated that this DNA intercalating drug does not affect the co-immunoprecipitation of hRPA with RECQ1 (Fig 8, lane 3). This result indicates that the interaction between RECQ1 and hRPA is not mediated by nucleic acids, as already observed by EMSA.

DISCUSSION

The detailed characterization of the substrate specificity and unwinding activity of the five human RecQ helicases as well as the discovery of protein partners that affect their enzymatic activity is essential for an understanding of the physiological function of this increasingly studied class of enzymes. In the present work, we have focused our attention on RECQ1, the first helicase of the RecQ family to be discovered in human cells, but also one of the less characterized in terms of its molecular and catalytic properties (48,49). For the first time, high yields of functional RECQ1 were produced in an insect expression system (48). The cDNA encoding for the 649 amino acid isoform of RECQ1 was amplified from human blood tissues, cloned and expressed in baculovirus, obtaining ~0.75 mg protein from 1.5 x 10^8 infected Sf9 cells. The helicase activity of our recombinant RECQ1 is comparable with that of RECQ1 purified from HeLa nuclear extract. Several helicases from different organisms have been identified and characterized to date (55,56). Some exist and function as monomers, others as dimers or hexamers (57,58). Different mechanisms for DNA unwinding have been proposed for monomeric, dimeric and hexameric helicases, respectively (59). Therefore, a knowledge of the oligomeric state of the helicase is crucial to understand the mechanism
that the enzyme adopts to translocate along the DNA molecule and to dissociate the base pairs during the unwinding process. The recently obtained crystal structure of the core domain of E.coli RecQ showed that this protein is a monomer in the absence of DNA (42). On the other hand, there is contradictory evidence regarding the oligomerization state of the full-length E.coli RecQ. A recent biophysical study on E.coli RecQ suggests that this enzyme functions as a monomer when unwinding DNA (23), in contrast to previous studies indicating that E.coli RecQ can form a multimer of at least three subunits in solution (22). The only information available so far on the oligomerization state of human RecQ helicases has been obtained for BLM, WRN and RECQ1. Electron microscopy and size exclusion chromatography experiments demonstrated that full-length BLM forms hexamers (60), while recent studies with the 642–1290 fragment of BLM revealed that BLM642–1290 runs as a monomer on a gel filtration column, both in solution and in its ssDNA-bound form (61). Similar gel filtration experiments suggested that the 333 amino acid N-terminal fragment of WRN as well as the full-length recombinant protein elutes as a trimer (62). Atomic force microscopy analysis of the 171 amino acid fragment of WRN, responsible for the exonuclease activity of this enzyme, revealed that this fragment is in a trimer–hexamer equilibrium in the absence of DNA and that this equilibrium is significantly shifted towards the hexamer in the presence of DNA (63). Our previous glycerol gradient and gel filtration studies on RECQ1 purified from HeLa cells indicated that the protein forms dimers in solution (20). On the other hand, the small amount of RECQ1 that we had available following its purification from HeLa nuclear extracts did not allow a careful investigation of the oligomerization state of the molecule in the presence and absence of DNA. Our new gel filtration results indicate that the recombinant RECQ1 has an apparent molecular mass of 158 kDa, in agreement with previous observations. To further support this conclusion and in order to see if the oligomerization state of RECQ1 may change in the presence of ssDNA, we performed DLS experiments both in the presence and absence of a 50 nt ssDNA fragment. The value of the hydration radius for RECQ1 is 5.4 ± 0.6 nm, compared to a hydration radius of 3.5 ± 0.4 nm for the BSA control, which has a molecular mass of 66 kDa. The higher value of the hydrodynamic radius confirms that RECQ1 does not exist as a monomer in solution. On the other hand, the value of the radius is higher than that expected for a simple RECQ1 dimer under a spherical shape assumption. This result could be consistent with a highly hydrated complex, a very elongated shape or an oligomeric complex formed by more than two subunits, although this last hypothesis is not supported by our gel filtration results. The value of the hydrodynamic radius does not change when a ssDNA fragment of 50 nt is added to the sample in the presence of 5 mM MgCl₂ and 5 mM ATP. Hence, the oligomerization state of RECQ1 does not change when the enzyme is bound to the 50 nt ssDNA probe. The fact that RECQ1 seems to form dimers rather than hexamers indicates that the five human helicases of the RecQ family may form different oligomeric structures and may adopt different mechanisms to unwind DNA.

Another key feature that characterizes DNA helicases is their substrate specificity. In this regard, the length of the duplex region that can be unwound varies dramatically among helicases. For example, the E.coli RecBCD is a highly processive helicase capable of unwinding >20 kb at a rate of 350 bp/s (64) and UvrD, another E.coli helicase, can unwind blunt ended duplex substrates of 2700 bp (65). In contrast, the human BLM and WRN helicases can only unwind double-stranded (ds)DNA fragments ≤53 and ≤91 bp, respectively (54,66). Similarly to BLM and WRN, our results show that RECQ1 can easily unwind dsDNA substrates shorter than 50 bp and less than 20% of a 110 bp duplex is unwound in the presence of 250 nM RECQ1.

On the other hand, considerably longer duplexes can be displaced by RECQ1 if hRPA is added to the reaction mixture. In fact, RECQ1 is able to unwind duplexes of 501 bp when hRPA is present. A possible explanation for the hRPA stimulation could be that it coats the single strand generated during opening of the duplex. Nevertheless, our data with ESSB show that only hRPA is able to stimulate the helicase activity of RECQ1, suggesting that hRPA performs an additional role in the unwinding reaction rather simply inhibiting reannealing of the displaced strand. The same specific effect of hRPA has already been observed for BLM and WRN that, in the presence of hRPA, can unwind DNA duplexes of 259 and 849 bp, respectively (24,25). The difference in number of base pairs that can be displaced by BLM, WRN and RECQ1 with the assistance of hRPA may be due to the different experimental conditions used. However, the observation that the unwinding activity of all these three helicases is specifically stimulated by hRPA suggests that they might all be involved in the physiological processes that require hRPA.

In order to shed light on the mechanism of hRPA stimulation, we tested the effect of hRPA on the ATPase activity of RECQ1. The \( k_{cat} \) value for the rate of ATP hydrolysis is 31 ± 2 min⁻¹ in the presence of a DNA fragment of 17 bp and increases to 126 ± 3 min⁻¹ in the presence of M13 ssDNA as effector (Table 2). These \( k_{cat} \) values are very similar to that measured for WRN, but 10-fold smaller than those determined for BLM and DmRECQ5 (11,24,25,61). In agreement with previous studies done with BLM and WRN (24,25), the presence of hRPA does not enhance the ATPase activity of RECQ1, suggesting that the molecular basis for the specific hRPA stimulation of the unwinding activity of these helicases must be different. Brosh and co-workers speculated that hRPA might help the recruitment of the helicase to the ssDNA–dsDNA junction in the ongoing helicase reaction (25). However, further studies with DNA substrates of different structures and with mutant proteins will be needed to reach a better understanding of the stimulation mechanism.

We demonstrated by far western analysis that RECQ1 physically interacts with the 70 kDa subunit of hRPA. The direct interaction between RECQ1 and hRPA is substantiated by the results of ELISA and co-immunoprecipitation experiments. ELISA assay with the purified proteins allowed the estimation of an apparent dissociation constant \( K_d = 6.2 \text{ nM} \) for the RECQ1–hRPA complex, close to that previously determined for the interaction of BLM with hRPA (25). The same experiment repeated using the DNA intercalating drug EtBr gives analogous results indicating that DNA is not mediating this interaction. Co-immunoprecipitation experiments with HeLa nuclear extracts proved that the
RECO1–hRPA complex is present in the cell nuclei and that the addition of EtBr does not disrupt the complex, confirming that nucleic acids are not required for the binding of RECO1 to hRPA. The BLM and WRN helicases also interact with hRPA, although the precise region of the helicases involved in the binding of hRPA and the functional role of this interaction remains an open question. Several proteins involved in different aspects of DNA metabolism have been shown to interact with WRN and BLM (12). Nevertheless, the precise location of the interaction domain within the respective RecQ helicase has been identified for only some of them (67). For example, the N-terminus of WRN contains a 3'-5' exonuclease domain and mediates the interaction of WRN with Ku70 subunit (27,68) and PCNA (34), while the C-terminus is responsible for the interaction with p53 (31,36), Ku80 subunit (27) and FEN1 (37). The extended N- and C-terminal domains of the BLM helicase mediate its interaction with topoisomerase III (38), MLH1 (39,40) and RAD51 (41). RECO1 lacks the extended N- and C-terminal tails of WRN and BLM, but contains the so-called RQC motif in its C-terminal tail. The shorter N- and C-terminal domains may explain why RECO1 is unable to interact with the two subunits of the Ku heterodimer. On the other hand, the fact that RECO1, BLM and WRN are all able to interact with hRPA suggests that the binding is mediated by a domain conserved among these three helicases. The RQC domain has been shown to be involved in the interaction between BLM and FEN1 (37) and to be required for the helicase activity of WRN (61). The binding of these helicases to hRPA may also involve the RQC domain. Alternatively, the motifs required for the helicase–hRPA interaction may be contained in the central helicase domain of 450 amino acids conserved among all RecQ helicases.

hRPA is a ssDNA-binding protein required for multiple processes in eukaryotic DNA metabolism, such as DNA replication, DNA repair and recombination (52). Therefore, BLM, WRN and RECO1 may also be involved in one or several of these processes. In particular, there is an increasing body of evidence suggesting that RecQ helicases are involved in the reinitiation of DNA replication at stalled replication forks, since it has been shown that they can remove potential ‘roadblocks’ such as G-quadruplex structures (17,69,70). The shared interaction with hRPA indicates that these RecQ helicases might be involved in common physiological processes and work in a complementary fashion such that the absence of any of them may be compensated for by the presence of another member of the family. On the other hand, only BLM is able to interact with RAD51 (41) and MLH1 (39,40), whereas WRN has the unique capacity of binding Ku (27) and PCNA (34), indicating that these helicases are also likely to be responsible for specific physiological functions. The growing attention on the RecQ helicases is also connected to their role as tumor suppressor genes, since it has been shown that mutations in RecQ helicase genes give rise to autosomal recessive disorders that are associated with an elevated incidence of cancer (3–5). Thus, the studies on the five human members of the RecQ family will be important to deepen our understanding of the DNA metabolic processes in which they are involved, as well as to improve our knowledge on the molecular basis of tumorigenesis.

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