THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 3, Issue of January 17, pp. 1424–1432, 2003
Printed in U.S.A.
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Characterization of the DNA-unwinding Activity of Human RECQ1, a Helicase Specifically Stimulated by Human Replication Protein A*

Received for publication, September 13, 2002, and in revised form, October 30, 2002
Published, JBC Papers in Press, November 4, 2002, DOI 10.1074/jbc.M209407200

Sheng Cui, Raffaella Klima, Alex Ochem, Daniele Arosio, Arturo Falaschi, and Alessandro Vindigni‡

From the International Centre for Genetic Engineering and Biotechnology, Padriciano 99, I-34012 Trieste, Italy

The RecQ helicases are involved in several aspects of DNA metabolism. Five members of the RecQ family have been found in humans, but only two of them have been carefully characterized, BLM and WRN. In this work, we describe the enzymatic characterization of RECQ1. The helicase has 3′ to 5′ polarity, cannot start the unwinding from a blunt-ended terminus, and needs a 3′-single-stranded DNA tail longer than 10 nucleotides to open the substrate. However, it was also able to unwind a blunt-ended duplex DNA with a "bubble" of 25 nucleotides in the middle, as previously observed for WRN and BLM. We show that only short DNA duplexes (<30 bp) can be unwound by RECQ1 alone, but the addition of human replication protein A (hRPA) increases the processivity of the enzyme (>100 bp). Our studies done with *Escherichia coli* single-strand binding protein (SSB) indicate that the helicase activity of RECQ1 is specifically stimulated by hRPA. This finding suggests that RECQ1 and hRPA may interact also *in vivo* and function together in DNA metabolism. Comparison of the present results with previous studies on WRN and BLM provides novel insight into the role of the N- and C-terminal domains of these helicases in determining their substrate specificity and in their interaction with hRPA.

DNA and RNA helicases are a ubiquitous class of enzymes characterized by their capacity to unwind and translocate along DNA or RNA in reactions that are coupled to the binding and hydrolysis of a 5′-NTP (1). These enzymes are involved in most aspects of nucleic acid metabolism, such as DNA replication, DNA repair, recombination, transcription, RNA processing, and translation (2). Alterations of genes that code for helicases cause several human disorders (3). For example, two genes, XPB and XPD, encode for helicases that are defective in individuals with xeroderma pigmentosum and Cockayne's syndrome, respectively (4). Bloom's, Werner's, and Rothmund-Thomson syndromes are additional genetic disorders that arise as a consequence of abnormalities in three different members of the RecQ family of helicases named BLM, WRN, and RECQ4, respectively (5–7). In all three syndromes, cells from affected individuals show inherent genomic instability, indicating that these RecQ helicases play a role in the maintenance of chromosome stability.

The name RecQ derives from the first helicase of the family discovered in *Escherichia coli* (8, 9). Successively, members of the RecQ helicase family have been found in organisms that range from bacteria to plants and animals (10, 11). In microorganisms like *E. coli*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*, only one representative per species is present, whereas higher eukaryotes contain more than one RecQ helicase. For example, five members of the RecQ family have been found so far in human cells, RECQ1, WRN, BLM, RECQ4, and RECQ5 (12). All of them share a common central domain of ~450 amino acids containing seven highly conserved motifs also present in several helicases from other families (13). Among these motifs are an ATP-binding sequence (Walker A box) and the DEXH box, which is instead characteristic of the RecQ family. The RecQ helicases are divided in two classes according to the length of the N- and C-terminal domains. *E. coli* RecQ, human RECQ1, and RECQ5 form the first group of RecQ helicases. They are characterized by short N- and C-terminal domains, and their sequences are between 400 and 650 amino acids long. The WRN, BLM, RECQ4, Sgs1p (from budding yeast), and Rqh1p (from fission yeast) helicases are part of the second group because they have extended N- and C-terminal tails and are all between 1300 and 1500 amino acids long. The function of these extended tails is still under investigation. One possibility is that the additional portions mediate the interaction of these helicases with other proteins. In fact, several proteins have been shown to interact with these longer helicases, such as replication protein A (14, 15), proliferating cell nuclear antigen (16), DNA topoisomerase I (16), Ku heterodimer (17–19), DNA polymerase δ (20), and p53 (21, 22). A common feature of all RecQ helicases studied so far is that they unwind DNA with a 3′ to 5′ polarity. On the other hand, only the DNA-unwinding activity and substrate specificity of the human BLM and WRN helicases have been thoroughly investigated (22–26), whereas little or no information is available so far on the catalytic properties of the other three human helicases, RECQ1, RECQ4, and RECQ5 (27, 28).

In this work, we describe the enzymatic characterization of the helicase activity of human RECQ1. The protein was purified from HeLa cells following a procedure similar to the one established in our laboratory for the purification of several other human DNA helicases (29–32). The possibility that the helicase activity of RECQ1 may be specifically stimulated by human replication protein A (hRPA),[1] as in the case of the Werner's and Bloom's helicases, was also investigated. Comparison of our results with the previously reported hRPA stimulation of the WRN and BLM helicases provides novel insight

* The work was supported by Grant 99.00649.PF33 from the Consiglio Nazionale delle Ricerche, Roma. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 39-040-375-7326; Fax: 39-040-226-555; E-mail: vindigni@icgeb.org.

[1] The abbreviations used are: hRPA, human replication protein A; ssDNA, single-stranded DNA; nt, nucleotides; BSA, bovine serum albumin; ESSB, *E. coli* single-strand binding protein.

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into the roles that the N- and C-terminal tails and the central 450-amino acid domain of these RecQ helicases play in the interaction with hRPA.

EXPERIMENTAL PROCEDURES

Reagents—All salts, bovine serum albumin, dithiothreitol, phenylmethylsulfonyl fluoride, leupeptin, and pepstatin were from Sigma. The M13mp18 single-stranded DNA (ssDNA) plasmid, the serum used to grow the HeLa cells, glutamine, and gentamycin were from Invitrogen. All resins used for the different purification steps were from Amersham Biosciences (Uppsala, Sweden). Most of the purification steps were carried out using an AKTA FPLC system (Amersham Biosciences). Gel filtration studies were performed with a TSK-GEL G3000SW column (60 cm × 7.5 mm; TOSOH BIOSEP, Stuttgart, Germany). All ssDNA oligonucleotides used to make the DNA substrates were purchased from Sigma (Cambs, UK). The radioactive nucleoside triphosphates were obtained from Amersham Biosciences (Buckinghamshire, UK). The T4 polynucleotide kinase and sequencing grade porcine trypsin for protein digestion were from Promega (Madison, WI). Recombinant hRPA containing all three subunits (RPA70, RPA32, and RPA14) was expressed in and purified from *E. coli* according to the previously described protocol (33).

Cell Culture and Buffers—HeLa cells were grown in Joklin minimal essential medium supplemented with 10% fetal calf serum, 50 μg/ml gentamycin, and 2 mM glutamine and harvested as described previously (31). All buffers used during the purification of RECQ1 contained 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride as protease inhibitor. Buffer A contained 20 mM HEPES (pH 8.0), 0.1 M NaCl, 1 mM EDTA, and 20% glycerol. Buffer B contained 50 mM Tris-HCl (pH 7.5), 70 mM KCl, 1 mM EDTA, and 10% glycerol. The concentration of NaCl or KCl in all buffers was increased up to 1.0 M for eluting the proteins from the different columns.

Purification of RECQ1—The RECQ1 helicase was purified from 300 g of frozen HeLa cells. The cell nuclei were isolated and salt-extracted with 0.4 M NaCl following the procedure described by Dignam *et al.* (34). Successively, an additional salt extraction with 1.0 M NaCl was done to select specifically for proteins that bind tightly to DNA. The extracted proteins were precipitated by slowly adding ammonium sulfate (0.35 g/ml), collected by centrifugation at 25,000 × *g* for 30 min in a Sorvall SS34 rotor, dialyzed in buffer A, and applied to a Bio-Rex column (2.5 × 33 cm) equilibrated with buffer A (35). Active fractions eluting at ~0.4 M NaCl in buffer A were pooled (Fraction I, 78 ml). All purification steps were carried out at 4 °C, and the unwinding activity after each step of purification was monitored with a 5′-^{32}P-labeled DNA substrate as described (29). Fraction I was first dialyzed in buffer B and then loaded onto a 10-ml Q-Sepharose column equilibrated with buffer B. The proteins bound to the column were eluted using a linear gradient of 0.07–1.0 M KCl in buffer B. All active fractions eluting at the very beginning of the gradient were pooled (Fraction II, 85 ml) and loaded onto a 1-ml Mono S column. Elution was carried out with the same linear gradient used for the Q-Sepharose column, and the helicase eluted between 0.2 and 0.3 M KCl (Fraction III, 14 ml). Fraction III was dialyzed in buffer B and loaded onto a 4-ml ssDNA-cellulose column (1.6 × 2.5 cm) for the last step of purification. Elution was carried out with a linear gradient of 5 column volumes of 0.07–1.0 M KCl in buffer B. A major peak eluted at ~0.24 M KCl. The active fractions were pooled together (Fraction IV, 8 ml, 44.800 units) and stored at −80 °C with 50% glycerol.

Preparation of DNA Helicase Substrates—The DNA substrates used in the helicase assay are listed in Fig. 5. They consist of different ^{32}P-labeled oligonucleotides annealed either to M13mp18 phage ssDNA or to ssDNA fragments of different lengths to create a partial duplex. The sequence of the 99-bp oligonucleotide used for the determination of the direction of unwinding (see Fig. 5, *A* and *B*) is the same as that described previously (36), as are the sequences of three oligonucleotides annealed to the M13mp18 phage ssDNA (see Fig. 5, *D–G*) (29, 36). The substrate in Fig. 5*H* was made using oligonucleotide 5′-CTCTAGAG-GATCCCCGGGTACCGAGCTCGAATT-3′ (33 bp), complementary to nucleotides (nt) 6231–6263 of M13mp18 phage ssDNA. The blunt-ended 25-bp duplex (see Fig. 5*C*) was made by annealing oligonucleotide 5′-GATCTCGCATCACGTGACGAAGATC-3′ to its complement. The substrate in Fig. 5*I* was made by annealing oligonucleotide 5′-GATCT-CGCATCACGTGACGATTTTTTTTTTTTTTTTTTTTTTTTTTGATCTC-GCATCACGTGACGA-3′ to a ssDNA fragment complementary to the oligonucleotide except for the 25 T stretch in the middle. Linear substrates with poly(dT) tails of different lengths were made using a ^{32}P-labeled 29-bp oligonucleotide (5′-GTCAAATAGCAAACATGAAAG-CATAAAAC-3′) annealed to complementary ssDNA fragments with 3′-tails of 10, 25, 50, and 75 dT nucleotides (see Fig. 5, *K–N*). The substrate with a double-strand region of 109 bp was 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 primer was annealed to region 114–137. PCR conditions were as follows: 10 mM Tris-HCl, 50 mM KCl, 0,1% Triton X-100, 2 mM $MgCl_2$, 200 μM dNTPs, 1 μM each primer, and 2.5 units of *Taq* polymerase (Promega) in a 50-μl reaction. Cycling conditions were as follows: initial denaturation at 95 °C for 2 min; 35 cycles at 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s; and finally, elongation at 72 °C for 7 min. For all substrates, 25 ng of each oligonucleotide, labeled at the 5′-end with T4 polynucleotide kinase and 0.9 MBq of [γ-^{32}P]ATP, were subsequently annealed to M13mp18 phage ssDNA (4 μg) in 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 50 mM NaCl. The mixture was heated at 95 °C for 2 min and slowly cooled to room temperature. Each substrate was purified by gel filtration through a 5-ml Sepharose 4B column.

Preparation of RNA/RNA Substrate—The RNA/RNA substrate was obtained as follows. The pDEST17 vector (Invitrogen) containing the annexin II gene was linearized by *Eco*RV and transcribed *in vitro* with T7 RNA polymerase (Promega) from the specific promoter, yielding a 2.2-kb RNA (30). A 16-bp RNA oligonucleotide complementary to the same region of annexin II (nt 53–68) was synthesized and labeled at the 5′-end with T4 polynucleotide kinase and 9.25 MBq of [γ-^{32}P]ATP. About 8 pmol of labeled oligonucleotide were mixed with 13 pmol of synthesized annexin II RNA, heated at 95 °C for 1 min, and allowed to anneal by slow cooling. The substrates were then purified by gel filtration through a 5-ml Sepharose 4B column.

DNA Helicase Assay—The helicase assay measures the unwinding of a γ-^{32}P-labeled DNA fragment from a partial duplex DNA molecule. The 10-μl reaction mixture contained 20 mM Tris-HCl (pH 9.0), 8 mM dithiothreitol, 2 mM $MgCl_2$, 3 mM ATP, 10 mM KCl, 4% (w/v) sucrose, 80 μg/ml bovine serum albumin (BSA), and ^{32}P-labeled helicase substrate (1000 cpm, 1 ng, ~0.05 nM). The helicase fraction to be assayed was added to the mixture and incubated at 37 °C for 30 min, and 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% nondenaturing polyacrylamide gel. The gel was dried, and the extent of DNA unwinding was quantitated by electronic autoradiography (Instant Imager, Packard Instrument Co.). One unit of DNA helicase is defined as the amount of enzyme unwinding 1% of the substrate in 1 min at 37 °C (30% in 30 min) in the linear range of enzyme concentration dependence.

Native Molecular Mass Determination—The native molecular mass was determined by glycerol gradient centrifugation and gel filtration analysis following the procedure described previously (29, 37, 38). More precisely, for the glycerol gradient study, 100 μl of 50 nM RECQ1 were layered on a 15–35% glycerol gradient in buffer B and centrifuged at 320,000 × *g* for 20 h at 4 °C. The standard protein markers were also run under the same conditions. The markers were catalase (240 kDa, 11.3 S), aldolase (158 kDa, 7.4 S), BSA (66 kDa, 4.22 S), and ovalbumin (45 kDa, 3.5 S). Fractions of 0.2 ml were collected from the top of the tube using an HSI Auto Densi-Flow IIC apparatus (Buchler Instruments) and assayed for helicase activity. For gel filtration, a TSK-GEL G3000SW column (60 cm × 7.5 mm) was used in the AKTA FPLC system equilibrated with buffer B. The solution of RECQ1 was first concentrated from 50 to 500 nM and then loaded onto the column. The column was run at a flow rate of 1 ml/min. Fractions of 0.25 ml were collected and assayed for helicase activity. The column was pre-calibrated using gel filtration molecular mass markers under the same conditions. The markers were thyroglobulin (670 kDa, Stokes radius of 85 Å), γ-globulin (158 kDa, 48.1 Å), BSA (66 kDa, 35.5 Å), ovalbumin (45 kDa, 30.5 Å), myoglobin (17 kDa, 21.2 Å), and vitamin B_{12} (1.35 kDa). The partition coefficient K_{av} is equal to $(V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the sample, V_0 is the void volume, and V_t is the total volume of the gel bed. The Stokes radius of RECQ1 was derived from the linear plot of $(-\log K_{av})^{1/2}$ *versus* the Stokes radius of the standard proteins. The molecular mass of RECQ1 was calculated from the Stokes radius and the sedimentation coefficient using the equation previously described (38).

Microsequence Analysis—The Coomassie Blue- and silver-stained bands containing RECQ1 were cut out and digested with bovine trypsin (Promega). The digestion products were separated by micro-high pressure liquid chromatography and analyzed by electrospray ionization mass spectrometry (Finnigan LCQ DECA, Thermo-Finnigan Corp., San Jose, CA).

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RESULTS

The human helicase RECQ1 was purified from the nuclear extract of HeLa cells following the purification steps described under "Experimental Procedures." The final product was loaded on a 10% SDS-acrylamide gel for analysis of its purity. The silver-stained gel showed only a single band of ~70 kDa (Fig. 1). Successively, the band was excised from the gel, digested with trypsin, and analyzed by mass spectrometry for protein identification. Ten peptides pertaining to the human DNA helicase RECQ1 (75 kDa) were found in the sample (Table I). The five helicases of the RecQ family that have been found in human cells are characterized by a conserved central domain of ~450 amino acids. On the other hand, the 10 peptides found by mass spectrometry have sequences that are unique for RECQ1, allowing us to be certain about the identity of the protein. Six peptides correspond to sequences located in the central domain of the protein, two in the N-terminal domain, and two in the C-terminal tail (Fig. 2).

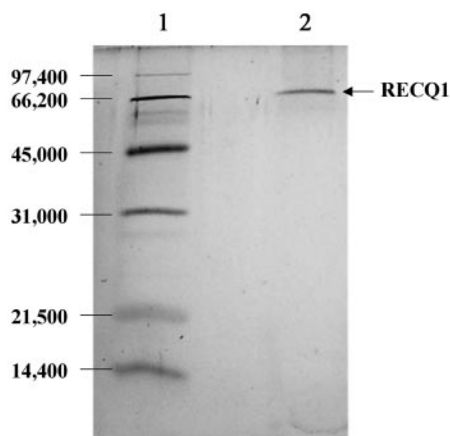

FIG. 1. **SDS-polyacrylamide gel of purified RECQ1.** *Lane 1*, molecular mass markers (in daltons); *lane 2*, purified RECQ1 (100 ng). The 10% acrylamide gel was stained with silver for better detection of eventual impurities. No additional bands were detected after loading 2 μg of RECQ1 on the gel.

After its identification, we determined the native molecular mass of RECQ1 by glycerol gradient and gel filtration following a previously described procedure (38, 39). The result indicates that RECQ1 has 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, thus suggesting that the protein exists as a dimer in solution (Fig. 3). On the other hand, further studies will need to be done under different buffer conditions and with additional techniques to obtain more accurate information on the oligomerization state of this molecule. Concentration dependence studies under optimal assay conditions showed a maximum value of ~100% unwinding in 30 min with 4 nM enzyme (Fig. 4*A*). The sigmoidal shape at the very beginning of the titration curve is indicative of cooperative behavior, suggesting that more than one molecule of RECQ1 could be involved in DNA unwinding, as seen in the case of other helicases (1). Kinetic measurements carried out in the presence of 1 nM enzyme (740 pg) showed that the unwinding rate was linear for up to 10 min and deviated from linearity with longer incubation times (Fig. 4*B*). The helicase assays indicated that ATP and Mg^{2+}

TABLE I
Amino acid sequences of the peptides present in RECQ1 identified by mass spectrometry

Positions	Sequence
32–38	QQELIQK
79–88	VKDILQNVFK
92–107	FRPLQLETINVTMAGK
186–193	LIYVTPEK
237–242	ALGILK
244–267	QFPNASLIGLTATATNHVLTDAQK
292–306	QKPSNTEDFIEDIVK
326–353	DSEQVTVSLQNLGIHAGAYHANLEPEDK
502–509	QAEELNEK
529–544	VAGVVAPTLPREDLEK

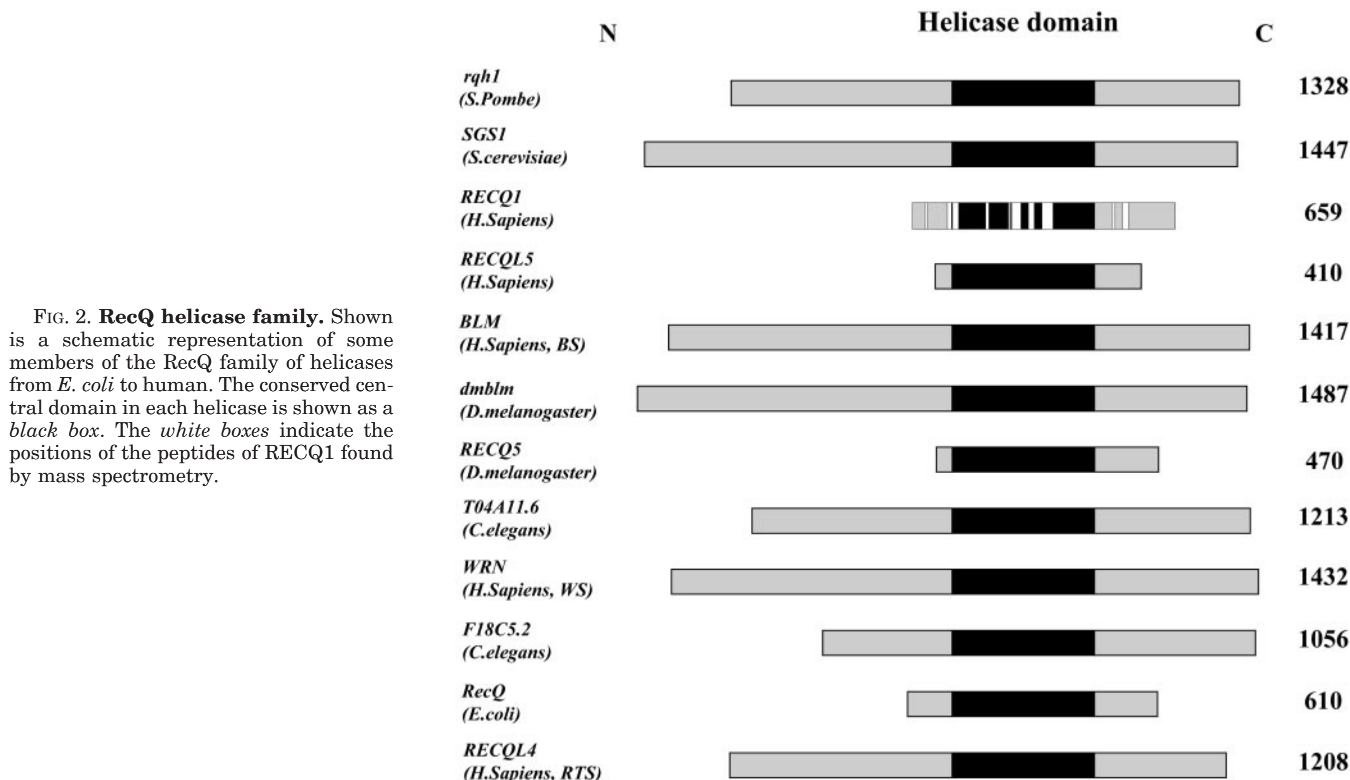

FIG. 2. **RecQ helicase family.** Shown is a schematic representation of some members of the RecQ family of helicases from *E. coli* to human. The conserved central domain in each helicase is shown as a *black box*. The *white boxes* indicate the positions of the peptides of RECQ1 found by mass spectrometry.

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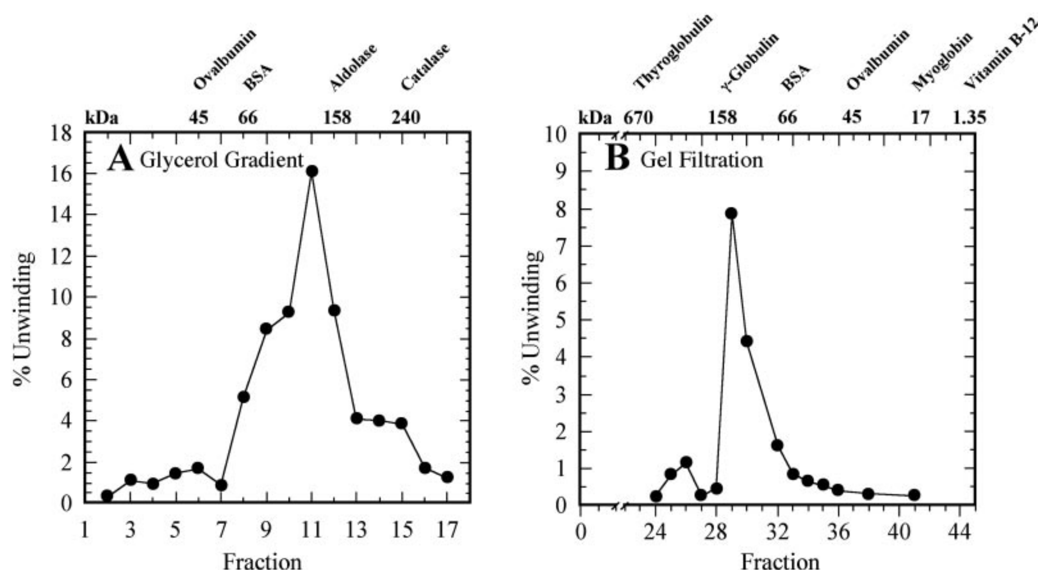

FIG. 3. **Glycerol gradient (15–35%) sedimentation and gel filtration analysis.** *A*, the experiment was performed using 100 μl of 50 nM RECQ1, and centrifugation was performed at 320,000 × *g* for 20 h at 4 °C. The distribution of the helicase activity, the positions of the sedimentation coefficients, and molecular mass markers are shown. The markers were catalase (240 kDa, 11.3 S), aldolase (158 kDa, 7.4 S), BSA (66 kDa, 4.22 S), and ovalbumin (45 kDa, 3.5 S). *B*, gel filtration was carried out using of 100 μl of RECQ1 (500 nM) on a TSK-GEL G300SW column (60 cm × 7.5 mm). The distribution of the helicase activity and the positions of the molecular mass markers are shown. The markers were thyroglobulin (670 kDa), γ-globulin (158 kDa), BSA (66 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa).

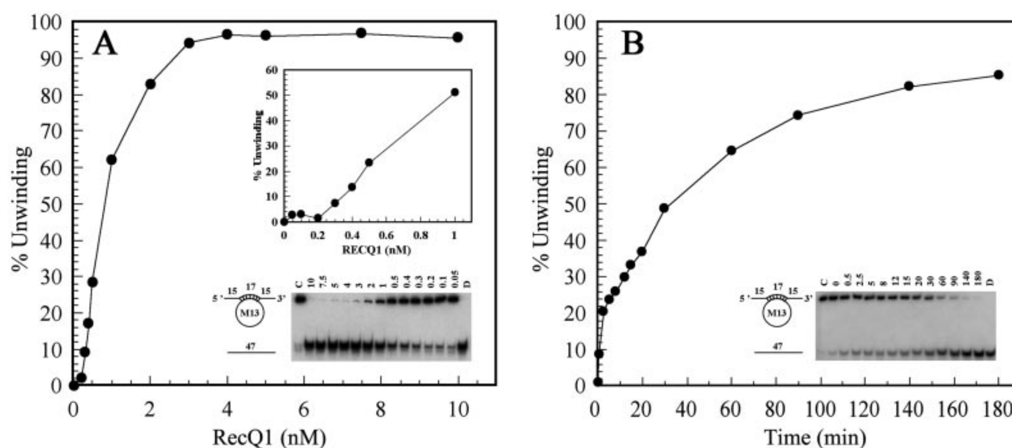

FIG. 4. **Concentration and time dependence of RECQ1 activity.** *A*, increasing amounts of RECQ1 were incubated in the standard 10-μl reaction mixture for 30 min at 37 °C. The concentration of enzyme (nanomolar) is indicated above each lane in the autoradiogram, whereas the substrate concentration was constant in all experiments (0.4 nM). *B*, the kinetics of unwinding were performed using 1 nM RECQ1 and the same reaction conditions used for the concentration dependence experiments. The reaction times (in minutes) are indicated above each lane in the autoradiogram. The *C* and *D lanes* are control assays without enzyme and heat-denatured substrate, respectively.

are indispensable for DNA unwinding. In addition, ATP dependence studies indicated that the optimal ATP concentration for DNA unwinding is between 4 and 5 mM (data not shown). Interestingly, we also observed that the addition of 80 μg/ml BSA increased the unwinding activity of RECQ1. This observation could be explained by previous studies showing that the presence of BSA increases the affinity of some proteins for DNA (40, 41).

The only information that was available on the helicase activity of human RECQ1 is that it unwinds DNA with 3′ to 5′ polarity, like the other members of the RecQ family characterized so far (42). This polarity of unwinding was confirmed by our experiments showing that RECQ1 needs a 3′-ssDNA tail to unwind the substrate (Fig. 5, *A–C*). Following this observation, a set of substrates with different structures and with various lengths of the double-strand regions were prepared to obtain novel information on the substrate specificity of RECQ1 (Fig. 5). Substrates with a 17-bp oligonucleotide annealed to M13 ssDNA were fully unwound by RECQ1, regardless of the presence or absence of mismatched hanging tails at the 5′-end, the 3′-end, or both (Fig. 5, *D–G*). However, if the duplex region was increased to 33 bp, the substrate could not be unwound (Fig. 5*H*). Similarly to what has been already observed for the BLM and WRN helicases (43), RECQ1 was able to unwind a substrate with a "bubble" of 25 nt located in the center (Fig. 5*I*). Other helicases previously purified in our laboratory have been shown to be able to unwind DNA/DNA as well as RNA/RNA duplexes (29); in contrast, RECQ1 could not unwind RNA substrates (Fig. 5*J*), indicating that it can work only as a DNA helicase. Finally, a series of linear substrates with 3′-single-strand tails of 10, 25, 50, and 75 dT were made to study the effect of tail length on the unwinding activity of RECQ1 (Fig. 5, *K–N*). Kinetic studies done with this series of substrates clearly showed that only the substrates with tails longer than 10 nt could be efficiently unwound by RECQ1 (Fig. 6).

hRPA specifically stimulates the DNA-unwinding activity of the WRN and BLM helicases (14, 15). In our experiments, we

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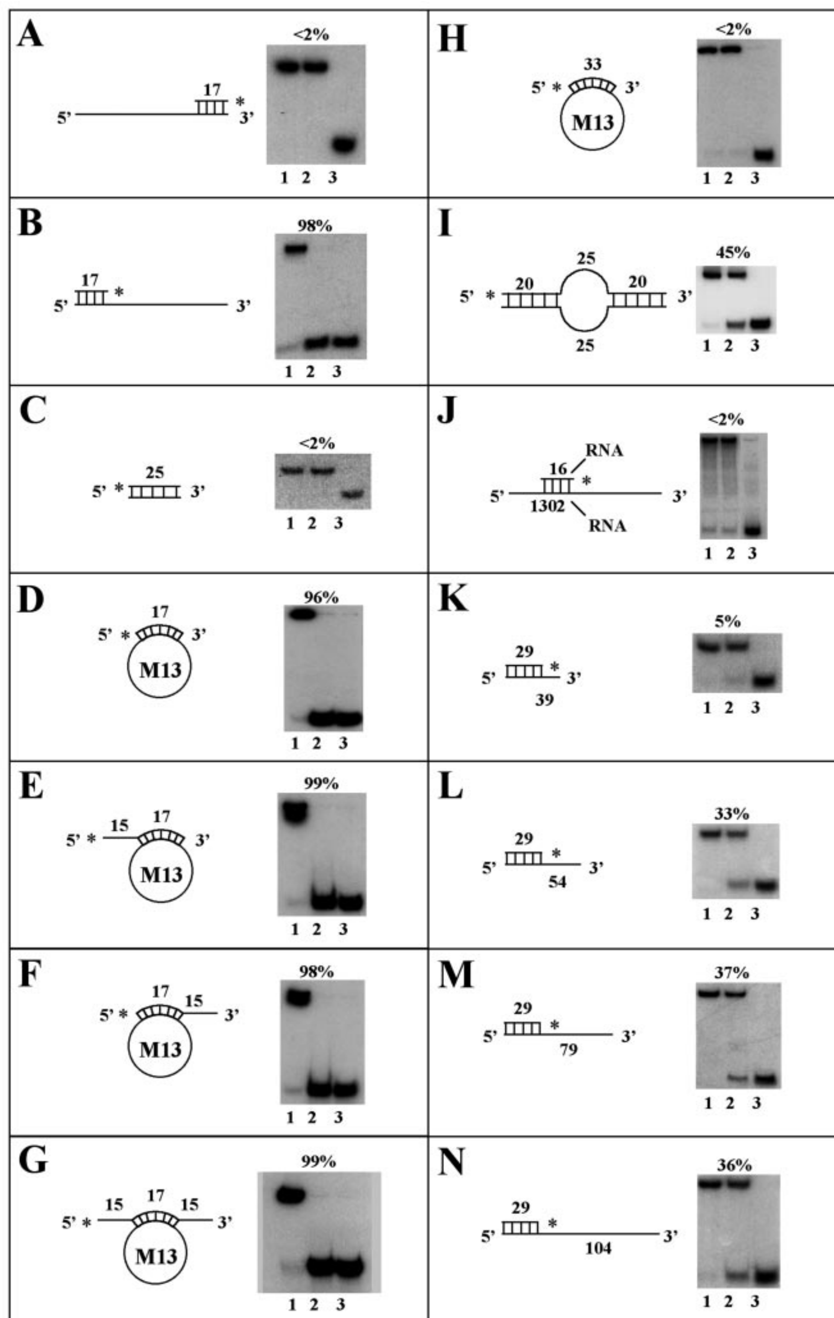

FIG. 5. **Helicase activity with various substrates.** Each panel shows the structure of the substrate used and an autoradiogram of the gel. *Asterisks* denote the ^{32}P-labeled end. Percent unwinding is shown above each autoradiogram. *Lanes 1* and *3* correspond to control reactions without enzyme and heat-denatured substrate, respectively. *Lane 2* corresponds to the reaction with pure RECQ1 (~1.0 nM). The direction of unwinding is analyzed in *A* and *B*.

saw that also the helicase activity of RECQ1 was specifically stimulated by the addition of hRPA. The RECQ1 helicase (1 nM) alone could not unwind a DNA substrate with a duplex region of 33 bp, but with the addition of hRPA or *E. coli* single-strand binding protein (ESSB), the substrate was unwound. Kinetic and concentration dependence studies showed that, in the presence of 0.3 μM hRPA, the unwinding reaction was complete in <15 min (Fig. 7, *A* and *B*). On the other hand, even at a concentration of ESSB (3 μM) 10-fold higher than that of hRPA (0.3 μM), only 40% of the substrate was unwound after 1 h. To gain more insight into the mechanism of stimulation by these single-strand binding proteins, strand displacement was expressed as a function of the ratio (*R*) of the concentration of single-strand binding protein units to the concentration of DNA-binding units (given by the concentration of the ssDNA substrate in nucleotides divided by the number of oligonucleotides covered by each unit). In this way, the new analysis takes into account that hRPA covers ~30 nt when binding to DNA (44), whereas ESSB binds ~35 nt (45) (Fig. 7*C*). The plot shows that, at a concentration of hRPA that coated the ssDNA molecules in the helicase reaction (96 nM heterotrimer), 15% of the 33-mer was unwound, whereas no unwinding was detectable at a coating concentration of ESSB (82 nM). Similarly, at an *R* value of 3, 100% of the duplex was released in the presence of hRPA, but no unwinding was observed with ESSB, suggesting that, even at high *R* values, ESSB poorly stimulates RECQ1 helicase activity. This observation was further supported by the results obtained with the 109-bp duplex (Fig. 8). This long substrate could be unwound only when hRPA was present, whereas ESSB failed to catalyze the unwinding even at a concentration 10-fold higher than that used for hRPA and after 3 h of reaction.

DISCUSSION

The physiological role of the five members of the RecQ family of helicases found in humans is still uncertain and

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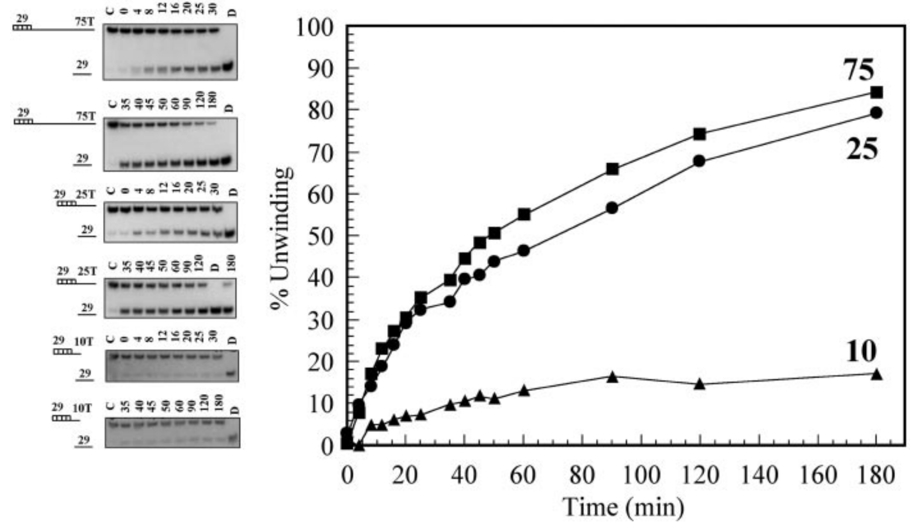

FIG. 6. **Tail length dependence studies.** The kinetics of unwinding were determined with three DNA substrates having poly(T) tails of 10 (▲), 25 (●), and 75 (■) nt. The concentration of RECQ1 used in all kinetic studies was 1 nM. The reaction times (in minutes) are indicated above each lane in the autoradiograms. The *C* and *D lanes* are control assays without enzyme and heat-denatured substrate, respectively.

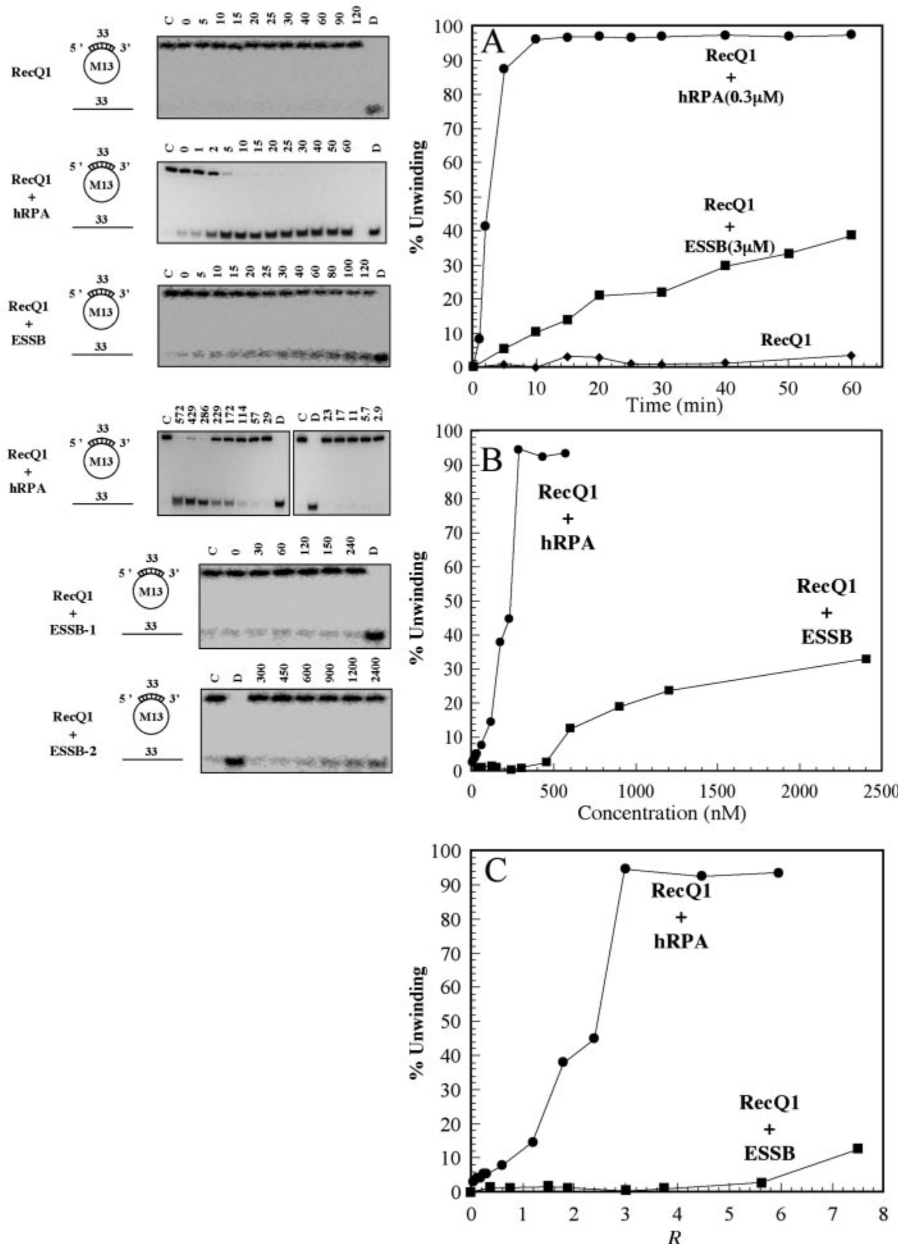

FIG. 7. **Stimulation of the helicase activity of RECQ1 by hRPA and ESSB.** The substrate used in all experiments was the 33-bp partial duplex shown in Fig. 5*H*, and the concentration of RECQ1 was always 1 nM. *A*, the kinetics of unwinding were determined using RECQ1 alone (♦) and in the presence of 0.3 μM hRPA (●) or 3 μM ESSB (■). The reaction times (in minutes) are indicated above each lane in the autoradiograms. *B*, concentration dependence studies were carried out at increasing concentrations of hRPA (●) and ESSB (■). The protein concentrations (nanomolar) are indicated above each lane in the autoradiograms. *C*, the percentage of unwinding is expressed as a function of *R*, defined as the ratio of the concentration of single-strand binding protein units to the concentration of DNA-binding units.

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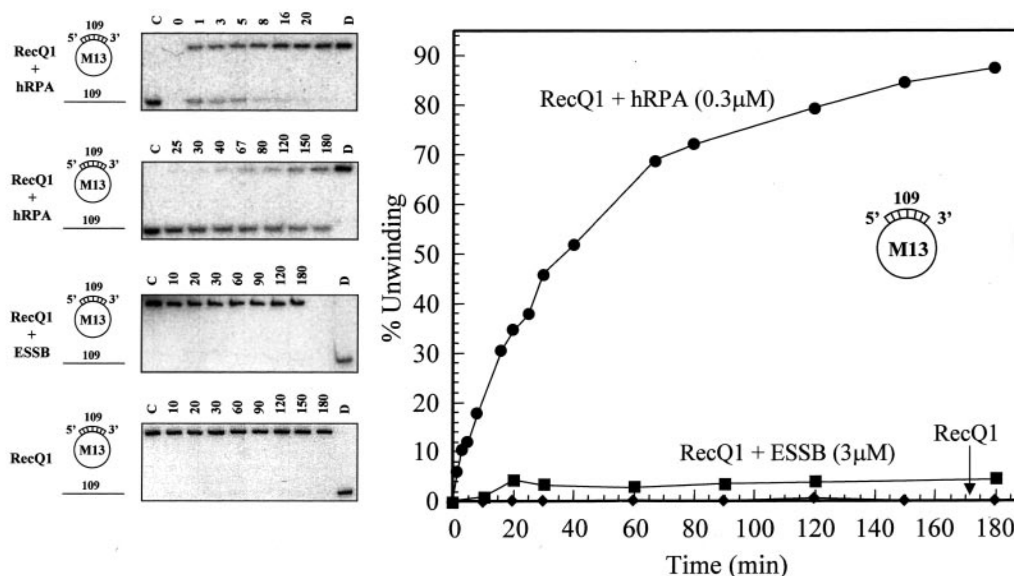

FIG. 8. **Kinetics of unwinding of a 109-bp partial duplex substrate in the presence of hRPA.** The kinetics of unwinding were determined using RECQ1 alone (♦) and in the presence of 0.3 μM hRPA (●) or 3 μM ESSB (■). The concentration of RECQ1 was always 1 nM. The reaction times (in minutes) are indicated above each lane in the autoradiograms.

under debate (12, 46). Several reports indicate that the human RecQ helicases play a key role in the maintenance of chromosome stability and point to their possible roles in different aspects of DNA metabolism, such as DNA replication, double-strand break repair, telomere maintenance, and homologous recombination (12, 46). In any case, deletion of the only RecQ family gene in *S. cerevisiae* leads to a significant impairment of DNA metabolism, but does not cause cell death (47). This also applies to the human cell mutants (48); but, in this case, it is conceivable that other members of the family partially rescue for possible essential function. Information on the substrate specificity of RecQ helicases and on their mechanism of DNA unwinding is essential for a better understanding of their function. On the other hand, only the enzymatic activity of two human RecQ helicases, WRN and BLM, has been carefully investigated to date (48). We isolated the human RECQ1 helicase from HeLa nuclear extracts and identified it by mass spectrometry; this is one of the first helicases of the RecQ family discovered in human cells, and very little information on its catalytic and molecular properties was available (49, 50). The only information available on the oligomerization state of the RecQ helicases comes from studies performed on the BLM protein, where it was shown, by electron microscopy and size-exclusion chromatography, that it forms hexameric structures in solution (51). On the other hand, our observation that RECQ1 seems to form dimers rather than hexamers suggests that the five helicases of the RecQ family may adopt different structures and may follow diverse mechanisms to unwind DNA. In addition, the sigmoidal shape of the titration curve as a function of increasing RECQ1 concentrations is indicative of cooperative behavior, suggesting that more than one molecule of RECQ1 could be involved in DNA unwinding, as seen in the case of other helicases (1); thus, the possibility that a hexameric structure builds up on the appropriate substrate cannot be ruled out.

The 3′ to 5′ polarity of unwinding was the only information available on the helicase activity of RECQ1 (42) and has been also confirmed by our results. The substrate specificity of RECQ1 was investigated with a series of DNA probes of different structures and lengths. As was previously observed for the BLM and WRN helicases (43), RECQ1 cannot unwind blunt-ended DNA substrates. In contrast, the *E. coli* RecQ helicase is able to initiate duplex unwinding from a blunt-ended terminus (52). Nevertheless, RECQ1 is able to unwind certain kinds of blunt-ended duplexes because it could unwind a blunt-ended DNA substrate with a 25-bp bubble in the center (Fig. 5), again in agreement with what had been previously observed for WRN and BLM (43). Based on the short size of its N- and C-terminal domains, RECQ1 is the human RecQ helicase, among the three (BLM, WRN, and RECQ1), that most resembles the *E. coli* variant. For this reason, it could have been predicted that RECQ1 would have greater similarity to *E. coli* RecQ than the other two human helicases. On the other hand, our findings suggest that the residues responsible for the substrate specificity of these enzymes must reside in the conserved central domain rather than in the N- and C-terminal domains and indicate that RecQ helicases from different organisms have different substrate specificities. We also studied the effect of the length of the 3′-ssDNA tails on the unwinding activity of RECQ1 (Fig. 5). Tail length studies have not been performed before on other human RecQ helicases, but it was shown that Sgs1p from *S. cerevisiae* is able to unwind substrates with 3′-tails of only 3 nt. Our results indicate that RECQ1 differs from Sgs1p because a DNA probe with a 3′-tail of 10 nt was poorly unwound and only when the tail length was increased to 25 nt >70% of the substrate was unwound (Fig. 5). A possible explanation is that RECQ1 needs a ssDNA tail longer than 10 nt to efficiently bind the substrate and to start the unwinding.

A common feature of the human RecQ helicases is that they are not able to unwind long DNA duplexes, but recent reports have shown that the addition of hRPA significantly enhances the processivity of WRN and BLM (14, 15). In line with these results, we observed that RECQ1 alone was unable to unwind a 33-bp duplex DNA probe under our experimental conditions, but the substrate could be easily unwound if hRPA was added to the reaction mixture. Upon addition of ESSB, the substrate could also be unwound, although much less efficiently because, even using 10-fold more ESSB than hRPA, only 40% of the duplex was unwound. The greater ability of hRPA over ESSB in stimulating the helicase activity of RECQ1 suggests that hRPA performs an additional role in

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the unwinding reaction rather than simply coating the single strands generated during the opening of the duplex. This conclusion is strengthen by the results obtained with the 109-bp partial duplex substrate. Helicase assays done in the presence of hRPA or ESSB showed that hRPA was absolutely required for the unwinding of the 109-bp duplex region, whereas ESSB was completely unable to stimulate the RECQ1-catalyzed unwinding reaction of this long substrate. These data indicate that both proteins, hRPA and RECQ1, are necessary for the unwinding of long substrates and suggest that these two proteins may functionally interact *in vivo*. A specific interaction between RECQ1 and hRPA could indicate a potential role of this helicase in replication, recombination, or repair, all processes in which hRPA has been shown to be involved (44).

Our findings also provide novel information on the roles of the N- terminal, C-terminal, and central domains of the RecQ helicases in the interaction with hRPA. Previous studies have shown that the N terminus of WRN contains a $3' \rightarrow 5'$ exonuclease domain and mediates the interaction of WRN with the Ku70 subunit (17, 19) and proliferating cell nuclear antigen (16), whereas the C terminus is responsible for the interaction with p53 (21, 53) and the Ku80 subunit (19). The extended N- and C-terminal domains of the BLM helicase mediate its interaction with topoisomerase III (54) and RAD51 (55). On the other hand, no information on the roles of the N- and C-terminal domains of these helicases in the interaction with hRPA is available. The observation that both WRN and BLM physically interact with hRPA could lead to the conclusion that the extended N- and C-terminal domains of these two helicases may also be involved in this binding event. However, our data suggest that the central domain of these RecQ helicases must be the one involved in the interaction of RECQ1, WRN, and BLM with hRPA because RECQ1 lacks the extended N- and C-terminal domains of WRN and BLM.

The picture that emerges from our studies is that the substrate specificity and DNA-unwinding activity of RECQ1 are, in many aspects, similar to those of the BLM and WRN helicases, although different from those of *E. coli* RecQ and budding yeast Sgs1p. Also the discovery that RECQ1 interacts with hRPA is in agreement with previous studies done with BLM and WRN. These similarities allow us to conclude that the extended N- and C-terminal domains of BLM and WRN are not responsible for the substrate specificity of these helicases and are not involved in the interaction of these proteins with hRPA. The fact that all three helicases interact with hRPA suggests that they could be involved in the same physiological processes and work in a complementary fashion, so the absence of one of them could be partially compensated for by the presence of the other. This hypothesis is also supported by other studies showing that WRN and BLM physically interact (23) and are both involved in DNA repair in a complementary fashion (56). On the other hand, the observation that WRN and BLM, with their extended N- and C-terminal domains, can also interact with other partners, such as p53, Ku, and topoisomerases, indicates that they can also be involved in some specific physiological functions where RECQ1 is not required. However, more studies will need to be done to reach a better understanding of the physiological roles of this intriguing family of human helicases.

Acknowledgments—We are indebted to Dr. Mark Wold for providing purified hRPA and the plasmid for the expression of hRPA in *E. coli*. The assistance of Maria Elena Lopez and Oscar Sandoval in the preparation of HeLa cells is gratefully acknowledged. We thank Marlen Lujardo Gonzàlez for assistance in the mass spectrometry analysis.

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Characterization of the DNA-unwinding Activity of Human RECQ1, a Helicase Specifically Stimulated by Human Replication Protein A

Sheng Cui, Raffaella Klima, Alex Ochem, Daniele Arosio, Arturo Falaschi and Alessandro Vindigni

J. Biol. Chem. 2003, 278:1424-1432.
doi: 10.1074/jbc.M209407200 originally published online November 4, 2002

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