The Human RecQ Helicases, BLM and RECQ1, Display Distinct DNA Substrate Specificities*[^5]

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RecQ helicases maintain chromosome stability by resolving a number of highly specific DNA structures that would otherwise impede the correct transmission of genetic information. Previous studies have shown that two human RecQ helicases, BLM and WRN, have very similar substrate specificities and preferentially unwind noncanonical DNA structures, such as synthetic Holliday junctions and G-quadruplex DNA. Here, we extend this analysis of BLM to include new substrates and have compared the substrate specificity of BLM with that of another human RecQ helicase, RECQ1. Our findings show that RECQ1 has a distinct substrate specificity compared with BLM. In particular, RECQ1 cannot unwind G-quadruplexes or RNA-DNA hybrid structures, even in the presence of the single-stranded binding protein, human replication protein A, that stimulates its DNA helicase activity. Moreover, RECQ1 cannot substitute for BLM in the regression of a model replication fork and is very inefficient in displacing plasmid D-loops lacking a 3’-tail. Conversely, RECQ1, but not BLM, is able to resolve immobile Holliday junction structures lacking an homologous core, even in the absence of human replication protein A. Mutagenesis studies show that the N-terminal region (residues 1–56) of RECQ1 is necessary both for protein oligomerization and for this Holliday junction disruption activity. These results suggest that the N-terminal domain or the higher order oligomer formation promoted by the N terminus is essential for the ability of RECQ1 to disrupt Holliday junctions. Collectively, our findings highlight several differences between the substrate specificities of RECQ1 and BLM (and by inference WRN) and suggest that these enzymes play nonoverlapping functions in cells.

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specificity of human RecQ helicases can provide valuable insights into the molecular basis of the different cellular functions of these enzymes.

A previous study showed that BLM and WRN have similar activity toward a panel of model DNA substrates of different structure and length, suggesting that, at least for these two enzymes, differences in helicase substrate specificity are not a fundamental distinguishing feature for defining their specific role in cellular DNA metabolism (23). In this study, we compared the substrate specificity of RECQ1 and BLM using a number of substrates of different structure and length, including substrates that have not been analyzed previously for either RECQ1 or BLM. Our findings highlight several differences between the enzymatic properties of RECQ1 and BLM and suggest that the role of RECQ1 in the maintenance of genome stability is distinct from that of BLM. The possible functions of these RecQ helicases in human cells are discussed.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Recombinant His<sub>6</sub>-tagged RECQ1 and BLM were expressed and purified following previously described procedures (28, 29). The RECQ1-(57–649) cDNA was PCR-amplified with a forward primer containing an Nhel site at the 5′-end and a reverse primer containing a XhoI site at the 5′-end. RECQ1-(57–649) cDNA was then cloned into Nhel and XhoI sites of a PET-28a(+) vector (Novagen), and the insert was excised using XbaI and XhoI in order to add the His<sub>6</sub> tag at the N terminus. The cDNA with the additional sequence for the His<sub>6</sub> tag was subcloned into the pFastBac1 vector using XbaI and XhoI. To obtain mutant RECQ1-(1–579), a termination codon after residue 579 was created by QuikChange XL site-directed mutagenesis kit (Stratagene). For the experiments with untagged RECQ1, the His<sub>6</sub> tag sequence was removed by directed mutagenesis kit (Stratagene). For the experiments with directed mutagenesis kit (Stratagene). For the experiments with the untagged RECQ1, the His<sub>6</sub> tag sequence was removed by digestion with thrombin (1:500 ratio) for 3 h at 4 °C in a buffer of 20 mM Tris-HCl, pH 7.4, 150 mM KCl, 5 mM β-mercaptoethanol. The sample was then incubated with the TALON metal affinity resin (Clontech) for 2 h at 4 °C. The flow-through containing the untagged RECQ1 was collected and concentrated using a Vivaspin filter (Vivascreen). The experiments with untagged BLM were performed with a truncated variant of the protein, BLM-(642–1290), expressed and purified from E. coli following previously described procedures (30).

**DNA Substrates**—All of the oligonucleotides used in this study are listed in Table 1. For each substrate, a single oligonucleotide was 5′-end-labeled with [γ-<sup>32</sup>P]ATP using T4 polynucleotide kinase. The kinase reaction was performed in PNK buffer (70 mM Tris-HCl, pH 7.6, 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol) at 37 °C for 1 h. [γ-<sup>32</sup>P]ATP-labeled oligonucleotides were then annealed to a 1.4-fold excess of the unlabeled complementary strands in annealing buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl) by heating at 95 °C for 6 min and then cooling slowly to room temperature. The purification of the forked duplex substrates was performed using Micro Bio-Spin columns (Bio-Rad) or ProbeQuant G-50 Micro columns (Amersham Biosciences). The G4 and G2′ DNA substrates carrying the consensus repeat from the murine immunoglobulin Sγ2b switch region or the Oxytricha telomeric repeat sequence were prepared as described previously (24, 25, 31). The Holliday

**TABLE 1**

| Number | Name | Sequence |
|--------|------|----------|
| 1      | Fork 30 (U) | TGGACCAGACCTAGCAGCTATGGGGGAGCTGGGGAAGGTGGGAATGTGA |
| 2      | Fork 30 (D) | TGGACCAGACCTAGCAGCTATGGGGGAGCTGGGGAAGGTGGGAATGTGATTTTTTTTTTTTTTTTTTCGTACCCGATGTGTTCGTTC |
| 3      | Fork 8 (U) | GAACGAACACATCGGGTACGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT |
| 4      | Fork 8 (D) | TGGACCAGACCTAGCAGCTATGGGGGAGCTGGGGAAGGTGGGAATGTGATTTTTTTTTTTTTTTTTTCGTACCCGATGTGTTCGTTC |
| 5      | G4-TP | TGGACCAGACCTAGCAGCTATGGGGGAGCTGGGGAAGGTGGGAATGTGA |
| 6      | G4-TP20 | TGGACCAGACCTAGCAGCTATGGGGGAGCTGGGGAAGGTGGGAATGTGATTTTTTTTTTTTTTTTTTCGTACCCGATGTGTTCGTTC |
| 7      | OX-1T | GACGCTGCCGAATTCTGGCTTGCTAGGACATCTTTGCCCACGTTGACCCG |
| 8      | X0-1 | GACGCTGCCGAATTCTACCAGTGCCAGCGACGGACATCTTTGCCCACCTGCAGGTTCACCC |
| 9      | X0-2 | GACGCTGCCGAATTCTACCAGTGCCAGCGACGGACATCTTTGCCCACCTGCAGGTTCACCC |
| 10     | X0-3 | GACGCTGCCGAATTCTACCAGTGCCAGCGACGGACATCTTTGCCCACCTGCAGGTTCACCC |
| 11     | X0-4 | GACGCTGCCGAATTCTACCAGTGCCAGCGACGGACATCTTTGCCCACCTGCAGGTTCACCC |
| 12     | X12-1 | GACGCTGCCGAATTCTGGCTTGCTAGGACATCTTTGCCCACGTTGACCCG |
| 13     | X12-2 | GACGCTGCCGAATTCTGGCTTGCTAGGACATCTTTGCCCACGTTGACCCG |
| 14     | X12-3 | GACGCTGCCGAATTCTGGCTTGCTAGGACATCTTTGCCCACGTTGACCCG |
| 15     | X12-4 | GACGCTGCCGAATTCTGGCTTGCTAGGACATCTTTGCCCACGTTGACCCG |
| 16     | X0-1 | GACGCTGCCGAATTCTACCAGTGCCAGCGACGGACATCTTTGCCCACCTGCAGGTTCACCC |
| 17     | X0-2 | GACGCTGCCGAATTCTACCAGTGCCAGCGACGGACATCTTTGCCCACCTGCAGGTTCACCC |
| 18     | X0-3 | GACGCTGCCGAATTCTACCAGTGCCAGCGACGGACATCTTTGCCCACCTGCAGGTTCACCC |
| 19     | X0-4 | GACGCTGCCGAATTCTACCAGTGCCAGCGACGGACATCTTTGCCCACCTGCAGGTTCACCC |
| 20     | X0-1 | GACGCTGCCGAATTCTACCAGTGCCAGCGACGGACATCTTTGCCCACCTGCAGGTTCACCC |
| 21     | X0-2 | GACGCTGCCGAATTCTACCAGTGCCAGCGACGGACATCTTTGCCCACCTGCAGGTTCACCC |
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junction substrates were purified using Sepharose-4B columns (Amersham Biosciences). The plasmid-based D-loop substrates were generated by RecA-mediated strand invasion of oligonucleotides DL3, DLm, and DL5 into pUC18 and were purified as described previously (32, 33). For the RNA-DNA heteroduplex, the radiolabeled rDL30m RNA oligonucleotide was annealed to pUCbottom in a 1:1.2 molar ratio, as described (33) and was used without purification. For the oligonucleotide-based R-loop, the RNA-DNA heteroduplex was further annealed to pUCtop in a 1:1.2 molar ratio and used without purification. The plasmid-based fork regression substrate was prepared as described previously by Ralf et al. (34). Briefly, the pG68 and pG46 plasmids that contain an array of BbvCI restriction endonuclease recognition sites were nicked by N.BbvC IA and N.BbvC IB, respectively. The two plasmids were annealed to create a paranemic joint, which was converted to a plectonemic joint by DNA topoisomerase I treatment to create the RF substrate molecule. The RF1 molecule was radiolabeled at the 3’ end using [α-32P]TTP and Klenow enzyme.

DNA Helicase Assays—Helicase assays were performed in 20 μl of a reaction mixture containing buffer A (20 mM Tris-HCl, pH 7.5, 8 mM dithiothreitol, 5 mM MgCl2, 10 mM KCl, 10% glycerol, 80 μg/ml bovine serum albumin), 5 mM ATP, and 32P-labeled helicase substrate (0.5 nM). These buffer and substrate concentrations were used in all unwinding assays, except where stated. For the G-quadruplex substrates, reactions were performed in buffer B (50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 50 mM NaCl, 100 μg/ml bovine serum albumin) using 5 mM ATP and 30 nM 32P-labeled helicase substrate, as described previously (25). The experiments with the plasmid-based D-loops, the RNA-DNA hybrids, and the R-loops were carried out in a 10-μl reaction volume in buffer C (33 mM Tris acetate (pH 7.8), 1 mM MgCl2, 66 mM sodium acetate, 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 1 mM ATP). When RNA-based molecules were used as substrates, RNase inhibitor (New England Biolabs) was added to the reaction to a final concentration of 2 units/μl. Recombinant RecQ helicase protein (RECQ1 or BLM) was added to a concentration indicated in the figures, and the mixture was incubated at 37 °C for the times specified in the figure legends. The reaction was terminated by the addition of 20 μl of 0.4 M EDTA pH 8.0, 10% glycerol (quench solution). Reaction products were resolved using 10% native PAGE, and the extent of unwinding was quantified as described previously (28). Fork regression assays were carried out as described previously (34).

ATPase and Electrophoretic Mobility Shift Assays—The ATPase and DNA binding assays were performed using procedures described previously (28). The rate of ATP hydrolysis was measured using thin layer chromatography assays. Reactions included different DNA probes at the concentrations indicated in the figure legends and 20 nM RECQ1 or BLM in buffer A. The electrophoretic mobility shift assays were performed in buffer B with various protein concentrations using 30 nM G-quadruplex DNA.

RESULTS AND DISCUSSION

The unusual ability of BLM and WRN to unwind cruciform and G-quadruplex DNA structures provides useful insights into the possible biological function(s) of these enzymes (23). The present study extends these observations, providing the first comparative analysis of the substrate specificity of RECQ1 and BLM (and by extension WRN). A series of oligonucleotide- and plasmid-based DNA substrates with different structures and lengths was generated to compare the substrate specificities of RECQ1 and BLM (Table 2). Initial experiments using forked duplex substrates with an ssDNA3 tail of 30 or 8 nt confirmed that both enzymes were catalytically active and were able to unwind fork-like structures with an ssDNA tail of ≥8 nt (supplemental Fig. 1) (data not shown). The similar specific activity of RECQ1 and BLM toward the forked duplex substrate was used to standardize each preparation of enzyme for the unwinding experiments using other DNA substrates. Moreover, all experiments were repeated using at least two independent preparations of each enzyme and under identical reaction conditions to minimize any possible interexperimental variation. An untagged version of RECQ1 was also used to exclude any possible contribution of the His6 tag in determining the substrate specificity of RECQ1 (supplemental Fig. 2). The results were consistent with our previous finding that His6-tagged and untagged RECQ1 have identical ATPase, unwinding, and strand annealing activities (35). Regarding BLM, previous studies have already demonstrated that a bacterially expressed (not His6-tagged) truncated variant of BLM, BLM-(642–1290), has the same substrate specificity as the full-length His6-tagged BLM isolated from yeast cells toward various linear duplexes, fork substrates, and Holliday junction structures (30). This result was also confirmed with some of the substrates used in this work (supplemental Fig. 2).

G-quadruplex DNA; a Putative Role of RecQ Helicases in the Removal of G4 DNA Structures from Ribosomal Gene Clusters, Immunoglobulin Heavy Chain Switch Regions, or Telomeric Repeats—Although G-DNA structures have not been unequivocally observed in vivo, the ease of formation of G-DNA in vitro suggests that G4 DNA structures probably exist at least transiently in cells (31). In particular, G-rich regions are abundant in ribosomal DNA gene clusters, in the immunoglobulin heavy chain switch regions, and within telomeric repeats. Although the complementary strand of a duplex would normally protect the G-rich strand from interstrand G-G pairing, cellular processes that promote DNA duplex unwinding, such as replication, transcription, or recombination, generate transient single-stranded DNA stretches that would allow G-quadruplex formation. Previous studies indicated that BLM and WRN could efficiently unwind G-quadruplex DNA substrates (21, 23, 25, 26, 36). Our experiments using different concentrations of BLM confirm this finding (Fig. 1A). Surprisingly, given that BLM, WRN, Sgs1, and E. coli RecQ can unwind G-quadruplex substrates (23–25, 34, 36), RECQ1 is not able to unwind this G4 structure even at RECQ1 concentrations of up to 500 nM or in the presence of the saturating concentration of the single-

3 The abbreviations used are: ssDNA, single-stranded DNA; nt, nucleotide(s); hRPA, human replication protein A; HJ, Holliday junction(s).
The same set of experiments was repeated using a G4 DNA substrate with a 3′-tail of 20 nt, with very similar negative results, indicating that the length of the tail does not affect the ability of RECQ1 to unwind this kind of DNA structure (Fig. 1, C and D). Again, BLM is able to disrupt this structure (Fig. 1, C and D). We also prepared a G2′ DNA substrate, containing the Oxytricha telomeric repeat sequence and a tail of 7 nt at the 3′-end, to test if RECQ1 could unwind this kind of G-quadruplex formed by two antiparallel hairpin dimers (24). Our data indicate that RECQ1 is unable to unwind G2′ DNA substrates (data not shown). In contrast, BLM can also efficiently unwind this substrate even at a protein concentration 200-fold lower.
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FIGURE 1. Analysis of the unwinding activity of RECQ1 and BLM using G-quadruplex substrates. A, unwinding experiments using increasing concentrations of RECQ1 (0–500 nM) or BLM (0–10 nM) and a G4 DNA substrate with a 3'-tail of 7 nt (30 nM). All of the reactions were stopped after 20 min. B, unwinding experiments in the presence of increasing concentrations of hRPA, as indicated, and 50 nM RECQ1. Lanes C and D represent controls without enzyme and with heat-denatured substrate, respectively. All of the reactions were stopped after 20 min. C, unwinding experiments using various concentrations of RECQ1 (0–200 nM) or BLM (0–20 nM) and a G4 DNA substrate with a 3'-tail of 20 nt (30 nM). Lanes C and D represent controls without enzyme and with heat-denatured substrate, respectively. All of the reactions were stopped after 20 min. D, quantification of the data from C. The data points represent the mean of three independent experiments with an S.D. value that was always ±10%.

FIGURE 2. ATPase and DNA binding assays using G4 DNA. A and B, thin layer chromatography assays performed using G4 DNA or oligo(dT)16 (32 μM) and 20 nM RECQ1 (A) or BLM (B) in the presence of increasing concentrations of ATP (5, 50, 100, 200, 400, and 800 μM). C and D, DNA binding assays at increasing protein concentrations using the G4 DNA probe and RECQ1 (C, 0.25, 0.5, 1, 2.5, 5, 10, 20, and 50 nM) or BLM (D, 0.25, 0.5, 1, 2.5, 10, 20, and 50 nM). The data points represent the mean of three independent experiments with an S.D. that was always ±10%. The insets show representative examples of the electrophoretic mobility shift assay using increasing protein concentrations.

To investigate the reason for the different unwinding activity of these two human RecQ helicases toward G4 DNA, we compared their ability to catalyze ATP hydrolysis in the presence of G-quadruplexes as co-factors. The calculated kinetic constant ($k_{cat}$) values for the ATP hydrolysis reactions catalyzed by BLM using G4 or ssDNA probes as DNA co-factors are 260 ± 10 and 330 ± 10 min$^{-1}$, respectively (Fig. 2A). The equivalent values for RECQ1 are 25 ± 2 and 91 ± 2 min$^{-1}$, respectively. These results suggest that the inability of RECQ1 to unwind this particular kind of DNA structure might be, at least partially, related to its poor ability to hydrolyze ATP in the presence of G-quadruplexes. Our gel mobility shift experiments showed, however, that RECQ1 efficiently binds G4 substrates with either a 7- or 20-nt 3'-tail with an affinity similar to that measured for the BLM protein (Fig. 2B). These results suggest that binding of RECQ1 to G4 DNA may not trigger the same conformational change necessary for the stimulation of the ATPase activity that is likely to take place with the BLM helicase.

Collectively, these findings point to a clear difference in substrate specificity between RECQ1 and all other members of the RecQ helicase family tested thus far, in that RECQ1 is unable to resolve G-quadruplex DNA structures. The fact that G-quadruplexes are generally the preferred substrate of BLM, WRN, Sgs1, and $E. coli$ RecQ indicates that G4 DNA may be a natural target for these helicases in vivo. In this regard, the formation of G-quadruplex structures may contribute to the genomic instability and hyperrecombination phenotypes characteristic of BLM-deficient cells. Our observation that RECQ1 does not unwind these structures indicates that this RecQ enzyme cannot substitute for BLM or WRN in the removal of G-quadruplex DNA structures and is likely to play a distinct function in cells.

RNA-DNA Hybrids; Potential Resolution of Transcription or Replication Intermediates—RNA-DNA hybrids are typical intermediates in the process of transcription and in the initiation of DNA replication. Increasing evidence suggests that a particular kind of RNA-DNA structure known as an R-loop can form as the result of transcription in cells harboring mutations in certain genes or when transcription results in long purine-rich stretches of RNA (37–42). The presence of these R-loops ahead of a translocating replication fork may induce the arrest
of DNA synthesis if these structures are not removed. Recent studies have shown that replicative helicases from different organisms, such as the *E. coli* DnaB, *Methanothermobacter thermautotrophicus* MCM, and the *Schizosaccharomyces pombe* Mcm4,6,7 complex unwind DNA-RNA hybrids by translocating along the ssDNA strand, suggesting that R-loops can be removed by these helicases to prevent DNA replication arrest (43). Interestingly, other studies have shown that R-loops may play a role during normal cell growth and may be critical for the maintenance of genome integrity (44–47). Moreover, following blockades of replication, R-loops may be required for the reassembly of a functional replication complex; the RNA at an R-loop would be extended by a DNA polymerase, opening up the duplex and leading to the recruitment of the replicative complex, thus allowing replication to restart while the original block is repaired or bypassed (45). To our knowledge, the ability of human RecQ helicases to unwind hybrid substrates containing DNA and RNA has not been investigated to date. Our data indicate that the BLM helicase could be involved in the removal of R-loops *in vivo*, since it can efficiently unwind a RNA-DNA hybrid substrate in which there are 43-nucleotide 3′ and 5′ DNA extensions (Fig. 3A). However, as with the G4 DNA substrates, RECQ1 cannot substitute for BLM in this function, since it is unable to unwind RNA-DNA hybrids even in the presence of saturating concentrations of hRPA (Fig. 3, B and C).

To study this second striking difference in substrate specificity between BLM and RECQ1 in more detail, we analyzed an oligonucleotide-based R-loop substrate in which an RNA oligonucleotide represented the “invading” strand. Previous studies have indicated that RecQ helicases could unwind such structures where all of the strands were composed of DNA (a D-loop) (16, 32, 48–50). Our previous analysis of D-loop unwinding by BLM indicated that the unwinding reaction occurred in two stages, where the “top strand” in Fig. 3 (which is not base-paired in the region of the “invading strand”) is first displaced, and then the remaining partial duplex is unwound, releasing the free, end-labeled “invading” strand (32). The data in Fig. 4A indicate that BLM can both remove the top strand and subsequently unwind the RNA-DNA hybrid to release a free RNA strand. In contrast, RECQ1 shows only a very low level of activity on this R-loop substrate (Fig. 4B). However, in the presence of 30 nM RPA, RECQ1 is able to efficiently disrupt the R-loop, although in this case, the reaction product is exclusively the RNA-DNA hybrid, representing only the removal of the top DNA strand. These data further confirm that RECQ1 is unable to unwind RNA-DNA hybrid molecules and highlight a second important difference in substrate specificity between RECQ1 and BLM, providing a strong indication that RECQ1 is not involved in the removal of R-loops *in vivo*.

**Plasmid-based D-loops: a Role in Resolution of Unproductive Recombination Intermediates?**—Another substrate that was recently shown to be among the preferred substrates of BLM is the plasmid-based D-loop (32). Previous studies have shown that several RecQ helicases, including RECQ1, are able to unwind oligonucleotide-based D-loops made of three partially complementary oligonucleotides (16, 48–50). These “static” D-loops are unlikely, however, to mimic D-loop structures generated during homologous recombination reactions *in vivo*, since they are not capable of branch migration and carry structural features, such as two free double-stranded DNA ends, that are not present in mobile D-loops *in vivo*. Our data indicate that, although BLM can efficiently displace plasmid-based D-loops with or without a protruding 3′ or 5′ ssDNA tail, RECQ1 can only effectively displace a plasmid D-loop with a 5′ invading terminus and a 3′ ssDNA tail (Fig. 5, A–C). Interestingly, our titration experiments at increasing protein concentrations indicate that RECQ1 is ~10-fold more active than BLM in displacing D-loops with a protruding 3′-end. Bachrati et al. (32) demonstrated that BLM resolves the “mobile” plasmid-based D-loop in a single step and suggested that this occurs through branch migration of the three-stranded junction, as would be predicted for a physiologically relevant mechanism of

FIGURE 3. Analysis of the unwinding activity of RECQ1 and BLM using RNA-DNA heteroduplexes. 0.1 nM RNA-DNA heteroduplex molecule, with a 3′ and a 5′ protruding DNA tail, was incubated with the indicated concentrations of BLM (A) or RECQ1 (B) for 15 min. C, as in B, except reactions contained 30 nM RPA. The sample loaded in lane 3 contained RPA only. Δ, heat-denatured substrate; C, mock-treated substrate.
expulsion of the invading ssDNA strand. A possible explanation for the fact that RECQ1 cannot efficiently displace plasmid D-loops without a 3’-OH tail is that RECQ1 cannot branch-migrate. Hence, only the D-loop with a 3’-tail can be unwound by RECQ1, because the protein can engage on the 3’ terminus and unwind the substrate in a “canonical” helicase reaction via denaturation of the duplex portion of the substrate. This 3’ terminus would not exist in vivo, because the invading strand in the recombination reaction would be far longer than we can mimic in vitro. D-loops with a 5’ invading strand may, however, be present in vivo, because the RAD51 protein can promote strand invasion from a 5’ ssDNA end as well as a 3’ ssDNA end (51). Such a structure represents an unproductive recombination intermediate, since DNA polymerases need a 3’-OH terminus on the invading strand to initiate DNA synthesis. Thus, D-loops with 5’ invading strand must somehow be prevented by an as yet unknown mechanism. Our data suggest that BLM could be involved in this process, providing an additional barrier to the formation of unproductive D-loops with a 5’ invading strand (32). RECQ1 would be unlikely to participate in such a reaction in vivo due to the lack of a 3’-end from which to initiate unwinding.

Model Replication Forks; a Putative Role in the Rescue of Stalled Replication Forks—Further indirect evidence in support of our hypothesis that RECQ1 cannot branch-migrate comes from the observation that RECQ1 does not promote the regression of a model replication fork in vitro (Fig. 6). Previous studies demonstrated that BLM uses its branch migration activity to mediate fork regression, generating regressed arms greater than 250 bp in length via the formation of a so-called “chicken foot” structure (34). This reaction is also catalyzed by some other RecQ helicases, such as WRN, but not by E. coli RecQ, and probably utilizes all of the known activities of RecQ helicases: strand separation, branch migration, and DNA strand annealing (52). The ability of BLM and WRN to promote fork regression suggests that BLM and WRN, unlike RECQ1 and E. coli RecQ, are involved in the rescue of stalled replication forks in vivo as part of a genome maintenance pathway. If replication forks stall as a result of encountering lesions on the leading strand template, the helicase-mediated fork regression reaction could facilitate template switching, allowing the leading strand to be extended past the lesion and replication to restart after the reversal of the regressed fork (34).

Holliday Junctions; the Processing of Homologous Recombination Intermediates—BLM and WRN can interact with Holliday junctions (HJ) and promote branch migration (20, 22). The ability of these two helicases to branch migrate HJ structures suggests that BLM and WRN might suppress hyperrecombination between sister chromatids and homologous chromosomes by disrupting potentially recombinogenic molecules that might arise at sites of stalled replication forks. Moreover, BLM is able to cooperate with topoisomerase IIIα and RMI1 to promote dissolution of double Holliday junctions (53). To test whether RECQ1 could catalyze Holliday junction disruption, we prepared a 50-bp-long synthetic HJ substrate (4-X12) that contains a 12-bp homologous core, following a previously described procedure (23, 54). Consistent with previous findings (23), recombinant BLM disrupts the X-junction into a splayed arm product and, to a lesser extent, into an ssDNA product using enzyme concentrations between 0.25 and 2 nM (Fig. 7A). At high BLM concentrations, the percentage of splayed arm product increases relative to that of the ssDNA product. A possible explanation for this phenomenon is that higher BLM concentrations are able to promote some reannealing of the ssDNA products, as reported previously (12). Analysis of RECQ1 protein indicates that it is less active than BLM toward the HJ substrate, since only a limited amount of either the splayed arm

![Graph](https://via.placeholder.com/150)

**FIGURE 4.** Analysis of the unwinding activity of RECQ1 and BLM using oligonucleotide-based R-loop substrates. The experimental procedure was identical to that outlined in the legend of Fig. 3, except for the use of a different substrate. The R-loop substrate was incubated with the indicated concentrations of BLM (A), RECQ1 (B), or RECQ1/RPA (C).
or the ssDNA product is observed at RECQ1 concentrations below 5 nM (Fig. 7B). These results were also confirmed by the use of kinetic experiments, which show that, although more than 60% of the HJ substrate is unwound within 10 min using 2 nM BLM, less than 50% of the X-junction is resolved into splayed arm or ssDNA products by 2 nM RECQ1 even after 60 min (Fig. 7C and D).

Interestingly, when we repeated the unwinding experiments using a 60-bp X-junction without a homologous core (4-X0), we found that BLM is very inefficient at disrupting this structure, whereas RECQ1 can unwind this 4-X0 substrate with an efficiency similar to that of the 4-X12 substrate (Fig. 8A). Kinetic experiments indicate that RECQ1 resolves this immobile HJ structure without formation of a splayed armed product intermediate, since the amount of splayed armed product detected during the course of the reaction is always very limited and much less than that seen in the experiments with the 4-X12 substrate (Fig. 8B). The BLM protein is only able to resolve these immobile HJ structures if hRPA is added to the reaction mix (Fig. 8C). Similar results were obtained with WRN. These findings suggest that RECQ1 disrupts HJ irrespective of the presence or absence of homology, possibly by using a mechanism that does not require branch migration. The fact that hRPA has a positive stimulatory effect on the activity of BLM suggests that this protein may bind the DNA in a particular mode that facilitates the resolution of HJ structures even in those cases where a preformed, branch-migratable junction is absent. Collectively, these findings point to an additional

4 V. Popuri and A. Vindigni, unpublished data.
important difference between BLM and RECQ1, in that whereas BLM can only unwind HJ structures lacking a homologous core in the presence of hRPA, RECQ1 is able to disrupt such an immobile X-junction even in the absence of hRPA. The different activities of RECQ1 and BLM toward HJ structures indicate that these two enzymes may play different roles in the resolution of HR intermediates in vivo.

Structural Domains Responsible for the Distinct Substrate Specificity of RECQ1 and BLM—The unwinding studies described above emphasize a number of significant differences between the substrate specificities of RECQ1 and BLM. The fact that RECQ1 is unable to resolve G-quadruplexes, RNA-DNA hybrid structures, and plasmid-based D-loops lacking a 3′ ssDNA tail or to substitute for BLM in the regression of model replication forks indicates that there must be some key structural features that distinguish the catalytic domain of RECQ1 from that of the other RecQ enzymes. For example, the previous observations that BLM, WRN, Sgs1, and E. coli RecQ can all unwind G4 substrates led to the conclusion that the domain responsible for this activity must be conserved among these proteins (23–25, 36). These studies, along with the observation that a mutant of the Saccharomyces cerevisiae Sgs1 helicase lacking the N- and C-terminal portions is still able to promote G4 DNA unwinding, led to the suggestion that the determinants necessary for the recognition and unwinding of G-quadruplex DNA must reside in the central “catalytic core” region of RecQ helicases comprising the helicase and the RecQ-conserved domains (24). DNA binding experiments with truncated forms of BLM confirmed that the RecQ-conserved domain of RecQ helicases is involved in the specific recognition of G4 DNA structures (36). However, RECQ1 contains the RecQ-conserved domain and yet is still unable to unwind G4 DNA. In this regard, the recent crystal structure of human RECQ1 shows that the relative position and orientation of the zinc-binding motif and the winged helix that form the RecQ-conserved domain of RECQ1 are different from those of E. coli RecQ (Protein Data Bank code 2V1X). Moreover, the RECQ1 structure shows a prominent β-hairpin, with a tyrosine residue at the tip, located in the wing of the winged helix domain that is much shorter in the structures of the winged helix domains of E. coli RecQ and WRN (55, 56). This hairpin might play an important role in DNA strand separation, as already suggested for other helicases of the SF2 family (57, 58), and in the definition the substrate specificity of RECQ1.

Other important domains for the distinct substrate specificity of RecQ helicases might reside in the N- and C-terminal regions of the proteins that have diverged in sequence between the different RecQ enzymes. For example, RECQ5β has a significantly shorter N-terminal domain compared with RECQ1 and BLM, exists as a monomer in solution, and has a reduced unwinding activity compared with RECQ1 and BLM, suggest-

FIGURE 7. Analysis of the unwinding activity of RECQ1 and BLM using a synthetic Holliday junction substrate with a 12-bp homologous core. Unwinding experiments using various concentrations of BLM (0–50 nM) (A) or RECQ1 (0–200 nM) (B). All of the reactions were stopped after 20 min. Lanes C and Δ represent controls without enzyme and with heat-denatured substrates, respectively. C, kinetic experiments using 2 nM BLM or RECQ1. All of the reactions were stopped at the times (min) indicated above the lanes. D, quantification of the data from C. The data points represent the mean of three independent experiments with an S.D. value that was always ≤10%. ◆, RECQ1; ○, BLM.

FIGURE 8. Analysis of the unwinding activity of RECQ1 and BLM using a Holliday junction substrate without a homologous core. A, plot of the unwinding activity as a function of protein concentration. The data points represent the mean of three independent experiments. ◆, RECQ1; ○, BLM. The inset shows an example of the unwinding assay using RECQ1 (2, 5, 10, 20, 40, 50, 80, 100, 150, and 200 nM) or BLM (2, 5, 10, 20, 40, 50, 80, 100, 150, and 200 nM). All of the reactions were stopped after 20 min. B, kinetics of DNA unwinding using 10 nM (◆) and 20 nM (○) RECQ1. The inset shows an example of the unwinding assay using 10 nM RECQ1 (the reaction was stopped after 1, 2, 4, 6, 8, 10, 12, 16, 20, 30, 40, 60, 90, and 120 min). C, unwinding experiments in the presence of increasing concentrations of hRPA and 2 nM (◆) RECQ1 or 20 nM (○) BLM. The inset shows an example of the unwinding assay using 2 nM RECQ1 or 20 nM BLM and various concentrations of hRPA (0, 1, 2, 5, 10, 20, 50, 100, 150, 200, and 300 nM). All of the reactions were stopped after 20 min. Lanes RPA and Δ represent controls with RPA alone and with heat-denatured substrate, respectively.
Substrate Specificity of RecQ Helicases

protein, the RECQ1-(57–649) protein elutes as a single peak corresponding to the smaller oligomeric form of RECQ1, suggesting that the N-terminal domain of RECQ1 is required for higher order oligomer formation (Fig. 9C). Collectively, our mutagenesis studies indicate that the N-terminal region, or the higher order oligomers formed as a result of its presence, is crucial for the HJ resolution activity of RECQ1. Interestingly, previous studies demonstrated that the same region is not required for the ability of BLM to disrupt HJ substrates, suggesting that the two enzymes use different domains to bind and resolve HJ structures (30).

Conclusions—Collectively, our findings show that RECQ1 has a distinct substrate specificity compared with BLM (and WRN), providing a strong indication that these helicases are likely to perform nonoverlapping functions in cells. Our results on the preference of RECQ1 for HJ substrates are consistent with a role of RECQ1 in HR repair. This would be also consistent with the recent finding that endogenous RECQ1 is associated with the strand exchange protein Rad51 and that depletion of RECQ1 results in spontaneous γ-H2AX focus formation and elevated sister chromatid exchanges (60). Previous studies indicated that BLM might be involved in both early and late steps of HR (32, 53). Several lines of evidence, including the results of this work, suggest that RECQ1 and BLM play different roles in the resolution of HR intermediates. In particular, BLM is the only human RecQ helicase able, in conjunction with topoisomerase IIIα and RM11, to promote the resolution of the so-called double Holliday junction structures that can form in the late step of HR (53, 61). Moreover, a specific function of RECQ1 in HR is supported by the analysis of embryonic fibroblasts from RECQ1-deficient mice that are hypersensitive to ionizing radiation and show an increased level of DNA damage and sister chromatid exchanges, indicating that the absence of RECQ1 cannot be compensated for by the presence of BLM (62). A recently published study indicates that endogenous DNA damage that remains unrepaired in cancer cells due to RECQ1 silencing induces cancer cell-specific mitotic catastrophe. Those authors suggest that RECQ1 might play an important role in the regulation of mitotic cell death in cancer and that this helicase might be a suitable target for the development of new chemotherapeutic agents (63). Although much progress has been made and it is now clear that RecQ enzymes do not simply play redundant roles in cells, the challenge of understanding the unique cellular function of RECQ1 in HR or in other DNA metabolic processes is still open.

FIGURE 9. Characterization of the enzymatic activity and oligomerization properties of the RECQ1-(1–579) and RECQ1-(57–649) mutants. A, top, unwinding experiments using wild type RECQ1 and the forked duplex (■), 4-X12 (○), and 4-X0 (△) substrates. Bottom, size exclusion chromatography profile of wild type RECQ1. The protein species were detected by protein fluorescence (λex = 290 nm and λem = 340 nm) following a previously described procedure (35). The peak at 9.5 ml corresponds to a calculated molecular mass of ~400 kDa, whereas the peak at 11.5 ml corresponds to a calculated molecular mass of ~155 kDa. B, top, unwinding experiments using different concentrations of RECQ1-(1–579) and the forked duplex (■), 4-X12 (○), and 4-X0 (△) substrates. Bottom, size exclusion chromatography profile of RECQ1-(1–579). C, top, unwinding experiments using different concentrations of RECQ1-(57–649) and the forked duplex (■), 4-X12 (○), and 4-X0 (△) substrates. Bottom, size exclusion chromatography profile of RECQ1-(57–649).

Unwinding experiments using a forked duplex substrate indicate that both mutants are able to fully unwind this substrate with an efficiency similar to that of the full-length protein (Fig. 9). However, the same experiments repeated using the two forms of HJ structure described above demonstrate that RECQ1-(57–649) is unable to resolve these structures regardless of the presence or absence of the homologous core sequence. RECQ1-(1–579) can still unwind these HJ substrates, even if to a somewhat lesser extent compared with the full-length RECQ1. Our previous studies demonstrated that RECQ1 exists in two quaternary structures: higher order oligomers consistent with a pentamer or a hexamer and a smaller species consistent with a monomer or dimer (35). Interestingly, size exclusion chromatography experiments indicate that, although the RECQ1-(1–579) mutant behaves like the wild type

ing a possible contribution of the N-terminal region in DNA unwinding (13). On the other hand, the C-terminal region of certain RecQ helicases contains an additional helicase-and-RNase D C-terminal domain that plays a role in DNA binding and is missing in RECQ1. Previous studies demonstrated that a key lysine residue, which resides in the helicase-and-RNase D C-terminal domain of BLM, is required for the double Holliday junction dissolution activity of this helicase (59). Moreover, a 60-amino acid region that lies adjacent to the helicase-and-RNase D C-terminal domain of BLM is essential for the strand annealing activity of BLM and might be also required for higher order oligomer formation (12). To test the functional and structural roles of the N- and C-terminal domains of RECQ1, we engineered two deletion mutants of RECQ1 lacking either the first 56 (RECQ1-(57–649)) or the last 69 (RECQ1-(1–579)) residues. Unwinding experiments using a forked duplex substrate indicate that both mutants are able to fully unwind this substrate with an efficiency similar to that of the full-length protein (Fig. 9). However, the same experiments repeated using the two forms of HJ structure described above demonstrate that RECQ1-(57–649) is unable to resolve these structures regardless of the presence or absence of the homologous core sequence. RECQ1-(1–579) can still unwind these HJ substrates, even if to a somewhat lesser extent compared with the full-length RECQ1. Our previous studies demonstrated that RECQ1 exists in two quaternary structures: higher order oligomers consistent with a pentamer or a hexamer and a smaller species consistent with a monomer or dimer (35). Interestingly, size exclusion chromatography experiments indicate that, although the RECQ1-(1–579) mutant behaves like the wild type
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