DNA Repair and Replication Fork Helicases are Differentially Affected by an Alkyl Phosphotriester Lesion*

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Background: Alkyl phosphotriester lesions are refractory to DNA repair.
Results: An alkyl phosphotriester lesion inhibits SF2 DNA repair helicases but not replication fork helicases or SF1 helicase UvrD.
Conclusion: Differences in the sensitivity of DNA helicases to an alkyl phosphotriester lesion suggest distinct unwinding mechanisms.
Significance: Alkyl phosphotriester lesions exert unique effects on DNA helicases, likely to have consequences for DNA metabolism.

SUMMARY
DNA helicases are directly responsible for catalytically unwinding duplex DNA in an ATP-dependent and directionally specific manner, and play essential roles in cellular nucleic acid metabolism. It has been conventionally thought that DNA helicases are inhibited by bulky covalent DNA adducts in a strand-specific manner. However, the effects of highly stable alkyl phosphotriester (PTE) lesions that are induced by chemical mutagens and refractory to DNA repair have not been previously studied for their effects on helicases. In this study, DNA repair and replication helicases were examined for unwinding a forked duplex DNA substrate harboring a single isopropyl PTE specifically positioned in the helicase translocating or nontranslocating strand within the double-stranded region. A comparison of SF2 helicases (RecQ, RECQ1, WRN, BLM, FANCJ, ChlR1) with a SF1 DNA repair helicase (UvrD) and two replicative helicases (MCM, DnaB) demonstrates unique differences in the effect of the PTE on the DNA unwinding.
reactions catalyzed by these enzymes. All the SF2 helicases tested were inhibited by the PTE lesion, whereas UvrD and the replication fork helicases were fully tolerant of the isopropyl backbone modification, irrespective of strand. Sequestration studies demonstrated that RECQ1 helicase was trapped by the PTE lesion only when it resided in the helicase translocating strand. Our results are discussed in light of the current models for DNA unwinding by helicases that are likely to encounter sugar phosphate backbone damage during biological DNA transactions.

A number of mutagenic and carcinogenic alkylating agents exist in the environment. In addition to modification of DNA bases, such chemical genotoxins can react with the oxygen atoms of the sugar phosphate backbone to form phosphotriester adducts (PTEs) (1). Esterification by covalent attachment of an alkyl moiety from an alkylating carcinogen to a non-carbon bonded oxygen results in the introduction of a hydrophobic group into the nucleic acid backbone and neutralization of the negatively charged phosphodiester moiety. The PTE configuration is dependent on which oxygen atom is esterified, potentially leading to an alkyl group that projects perpendicular to the double helix axis ($S_p$) or projects into the major groove ($R_p$). Evidence suggests that PTEs are chemically stable under physiological conditions, persist in DNA long after cellular exposure to the alkylating agent, and can inhibit processes of DNA repair by affecting the catalytic functions of DNA metabolic enzymes such as certain DNA polymerases, and endo- or exonucleases.

The inhibitory effect of PTEs on DNA repair systems was of interest to us because a number of Superfamily (SF)1 and SF2 DNA helicases, defined by their amino acid sequence homology within the helicase core domain (2), are implicated in various DNA repair pathways. For example, a key DNA helicase in *E. coli* that is required for nucleotide excision repair (NER) and methyl-directed mismatch repair is the SF1 UvrD enzyme, also known as DNA helicase II (3). SF2 DNA helicases have achieved prominence because a number of them have been implicated in hereditary diseases characterized by chromosomal instability and defects in DNA repair. These would include the RecQ family members (WRN, BLM, and RECQL4) that are genetically linked to Werner syndrome, Bloom’s syndrome, and Rothmund-Thomson syndrome, respectively, and two others, RECQ(L)1 and RECQ(L)5 (4-5). Although RECQ1 and RECQ5 have not yet been implicated in a hereditary disorder, they are also important for maintenance of genomic stability (6-8). The SF2 helicase family also includes the Iron-Sulfur (Fe-S) cluster helicases FANCJ and ChlR1 which are genetically linked to Fanconi Anemia (FA) (9) and Warsaw Breakage syndrome (WABS) (10), respectively. FANCJ is thought to have a role in the recombination repair phase after interstrand cross-link (ICL) processing, whereas ChlR1 is involved in the suppression of sister chromatid cohesion and resistance to DNA cross-linking agents and topoisomerase I inhibitors by a not well understood mechanism. In addition, mutations in the Fe-S helicase XPD that is implicated in eukaryotic NER and transcription can give rise to Xeroderma pigmentosum (XP), XP combined with Cockayne’s syndrome, or Trichothiodystrophy (11).

From a biochemical standpoint, the SF1 and SF2 DNA helicases are believed to function by different mechanisms; however, the details are only beginning to emerge (12). For SF1 helicases, the major contacts between the protein and DNA are with the bases; however, electrostatic interactions with the sugar phosphate backbone also exist. An unwinding mechanism known as the ‘Mexican Wave’ has been described for a SF1 helicase PerA in which DNA bases are flipped out as they make hydrophobic contacts with aromatic amino acids of the helicase (12). Analysis of SF1 UvrD crystal structures provided evidence that UvrD unwinds one base pair at a time per ATP bound, followed by translocation on the emerging single strand with ATP hydrolysis (13).

Auxiliary domains in addition to the conserved helicase motifs are likely to play a
role in the DNA unwinding reaction for SF1 and SF2 helicases. For example, RecQ helicases contain a RecQ C-terminal region (RQC) located just after the helicase core domain that mediates interaction with DNA and other proteins (4,14). The Fe-S helicases contain a conserved Fe-S cluster and an Arch motif that together form an Arch domain that is proposed to channel one strand of the unwound duplex (15-18). For SF2 helicases, the major contacts are thought to be with the phosphodiester backbone through ionic interactions with the helicase protein; however, based on the recently solved XPD-DNA crystal structure and biochemical characterization of site-directed mutants, it was speculated that certain aromatic residues near the Fe-S domain of the SF2 helicase could be involved in stacking interactions with DNA bases (16).

In addition to the DNA repair helicases, ring-like helicases operate at the replication fork to mediate unwinding of the parental duplex to provide the single-stranded DNA template for DNA synthesis by the polymerase. In *E. coli*, the SF4 DNA helicase DnaB unwinds forked duplex DNA substrates by translocating 5' to 3' with respect to the strand that the hexameric helicase is bound with the unwound 3' single-strand extruded outside the central channel of the protein (19). In eukaryotes (20) and archaea (21), MCM, belonging to the AAA+ SF of ATPases, is the replicative ring-like helicase that unwinds duplex DNA with a 3' to 5' polarity. A significant area of interest in cellular replication of damaged DNA is the signaling mechanism established by uncoupling of the replicative helicase duplex unwinding at the replication fork from DNA polymerase synthesis which can be delayed or blocked by a base or sugar phosphate backbone lesion (22).

Mechanistic and biological aspects of helicase action on damaged DNA has become a topic of considerable interest (23). However, to our knowledge there has been no previous report on the biochemical effect of sugar phosphate backbone alkylation damage on the unwinding reaction catalyzed by a DNA helicase. Given the potential mutagenic and carcinogenic consequences of such lesions, we thought it was important to evaluate the impact of a defined alkyl modification on helicase function. In this study, we have investigated the effects of a single isopropyl PTE (Scheme 1) specifically positioned in the helicase translocating or nontranslocating strand within the double-stranded region of a forked duplex DNA substrate. A comparison of a SF1 DNA repair helicase (UvrD), SF2 helicases (RecQ, RECQ1, WRN, BLM, FANCJ, ChlR1) and replicative helicases (MCM, DnaB) demonstrates unique differences in the effect of the PTE on the DNA unwinding reactions catalyzed by these enzymes. Our results are discussed in light of the current models for DNA unwinding by these helicases that are likely to encounter PTEs during biological DNA transactions.

**EXPERIMENTAL PROCEDURES**

**DNA Strates**— The isopropyl phosphotriester oligonucleotides, which were prepared by standard automated phosphoramidite synthetic procedures using commercially available reagents, were a gift from Matthew Swenson and Paul Miller, Department of Biochemistry and Molecular Biology, Johns Hopkins Bloomberg School of Public Health. PAGE-purified oligonucleotides used for the preparation of DNA substrates are listed in Table I. DNA duplex substrates were 5'-32P-end-labeled and prepared as described previously (24).

**Recombinant Helicase Proteins**— Recombinant human FANCJ (25), ChlR1 (26), RECQ1 (27), and WRN (28) were purified as described previously. *E. coli* DnaB (29) and *M. thermoautotrophicum* MCM (30) were purified as described. *E. coli* UvrD was purified as previously described (31). *E. coli* RecQ was purchased from Abcam. Human recombinant BLM protein was kindly provided by Dr. Ian Hickson (University of Copenhagen).

**Helicase Assays**— Standard helicase reaction mixtures (20 µl) containing 10 fmol of the specified forked duplex DNA substrate (0.5
nM DNA substrate concentration), and the indicated concentrations of the specified helicase were performed using previously described reaction conditions (FANCJ (32), WRN (24), RECQ1 (27), BLM (33), MCM (30), DnaB (29), UvrD (34), and ChlR1 (26)). Unless specified otherwise, DNA unwinding reactions were initiated by the addition of helicase and incubated for 15 min. Reactions were quenched with the addition of 20 µl of 2x Stop buffer (17.5 mM EDTA, 0.3% SDS, 12.5% glycerol, 0.02% bromophenol blue, 0.02% xylene cyanol). A 10-fold excess of unlabeled oligonucleotide with the same sequence as the labeled strand was included in the quench to prevent reannealing. The products of the helicase reactions were resolved on non-denaturing 12% (19:1 acrylamide:bisacrylamide) polyacrylamide gels. Radiolabeled DNA species in polyacrylamide gels were visualized using a PhosphorImager and quantitated using the ImageQuant software (Amersham Biosciences). The percent helicase substrate unwound was calculated by using the following formula: % Unwinding = 100 x (P/(S + P)), where P is the product and S is the substrate. The values of P and S have been corrected after subtracting background values in the no enzyme and heat-denatured substrate controls, respectively. Helicase data represent the mean of at least three independent experiments with standard deviation (SD) shown by error bars.

RESULTS

The DNA substrates used for this study are composed of specifically designed oligonucleotides that contain a single isopropyl PTE lesion (Scheme 1) positioned in either the top or bottom strand within the duplex region of a forked duplex substrate (Table 1). There are 12 bp residing between the 19 nucleotide (nt) single-stranded tails and the site of the PTE, and 12 bp residing between the PTE and blunt duplex DNA end on the opposite end of the forked duplex substrate. As a control, a forked duplex of identical sequence without any modification in either strand was tested for DNA unwinding by various helicases.

Helicase Sequestration Experiments—For helicase sequestration studies, RECQ1 (7.8 nM) was preincubated with the indicated concentrations (0 – 12.5 nM) of the specified unlabeled forked duplex competitor DNA molecule harboring an isopropyl PTE lesion in either the top strand, bottom strand, or neither strand in standard helicase reaction buffer in the presence of ATP (2 mM) for 3 min at 30 °C. Ten fmol of radiolabeled forked 19 base pair (bp) duplex molecules (tracker substrate (35)) was subsequently added to the reaction mixture and incubated for 7 min at 30 °C. Reactions were quenched and resolved on native polyacrylamide gels as described above. Percent helicase substrate unwound was calculated as described above. Typically, 90% of the tracker substrate was unwound in reactions lacking competitor DNA molecule. Helicase data (% control) were expressed relative to the control reactions lacking the competitor DNA.

Inhibition of human RECQ1 helicase by the isopropyl PTE lesion

Initially, we tested the human 3’ to 5’ RECQ1 helicase on a set of DNA fork substrates (Table 1) containing an isopropyl PTE backbone modification positioned in
either the top (nontranslocating) or bottom (translocating) strand. The control undamaged DNA substrate or substrate with PTE damage in the top strand (PTE-C) was unwound by RECQ1 in a protein concentration-dependent manner through out a titration range of 0.03 to 17.6 nM RECQ1 monomer (Fig. 1A and B). Eighty percent of substrate was unwound at 5 nM RECQ1. At higher concentrations of RECQ1, more substrate was unwound; however, the percent unwinding began to plateau, presumably due to substrate depletion effects. Significantly lower RECQ1 helicase unwinding was observed for the forked duplex substrate containing the isopropyl PTE damage in the bottom (translocating) strand (PTE-B) compared to the forked duplex substrate with the alkyltriester lesion in the top strand or control DNA substrate without any damage. For instance, only 28% of the substrate with the PTE lesion in the translocating strand was unwound at 5 nM RECQ1 compared with approximately 85% of the control substrate or substrate with the PTE in the top (nontranslocating) strand. Quantitation of helicase product unwound at the lower RECQ1 protein concentrations (inset) showed a modest inhibition of RECQ1 by the PTE in the nontranslocating strand; however, it was evident that this inhibition could be overcome by higher RECQ1 protein concentrations.

To further examine the effect of the PTE lesion on RECQ1 helicase activity, we performed a kinetic analysis using the same concentration of DNA substrate (0.5 nM) as that used for the RECQ1 protein titration experiments, but a fixed RECQ1 concentration of 18 nM (Fig. 1C). Initial rates of DNA unwinding were determined through 3 min of incubation. This rate analysis demonstrated that RECQ1 helicase activity was reduced approximately 5-fold by the translocating strand lesion compared to the control substrate, and less than 2-fold by the nontranslocating strand lesion (Fig. 1D).

The greater but still incomplete inhibition of RECQ1 helicase activity by the PTE damage when the lesion was positioned in the translocating strand suggested that the multi-turnover conditions may provide RECQ1 the opportunity to overcome the obstacle and unwind the entire duplex. To disfavor this situation, we increased the DNA substrate 10-fold (final concentration 5 nM) and kept the RECQ1 protein concentration (18 nM) the same (Fig. 1E). RECQ1 and DNA substrate were preincubated for 3 min at 24 °C followed by simultaneous addition of ATP and a large excess of dT120 (500 nM) to dissuade excess RECQ1 molecules from loading on to forked duplex substrates that were only partially unwound by RECQ1 during the course of the 40 sec reaction time. Under these conditions, we observed for all three substrates there was an increase in product as a function of time throughout the time course of the experiment (Fig. 1F). RECQ1 unwound significantly less of the DNA substrate with the PTE lesion in the translocating strand compared to the control undamaged substrate. The substrate with the nontranslocating strand PTE was unwound slightly less efficiently than the control substrate, consistent with the rate analysis (Fig. 1D) and RECQ1 protein titration data (Fig. 1B).

**RECQ1 is sequestered by the translocating strand PTE lesion**

The stronger inhibition of RECQ1 helicase activity by a PTE lesion in the translocating strand suggested that during unwinding RECQ1 might become sequestered. To address this issue, protein trapping experiments were performed to evaluate if RECQ1 was sequestered differentially during unwinding of DNA molecules containing the isopropyl triester linkage in the translocating versus nontranslocating strand. If sequestration of RECQ1 occurs by the PTE damage, preincubation of RECQ1 in the presence of ATP and the unlabeled DNA substrate containing the PTE lesion in the translocating strand should trap the RECQ1 helicase and prevent it from unwinding a radiolabeled control forked duplex tracker substrate subsequently added to the reaction mixture (Fig. 2A). Increasing concentrations of unlabeled forked duplex molecules containing the PTE in the translocating strand, nontranslocating strand, or neither strand was pre-incubated with RECQ1 (8.7 nM) for 3 min.
in the presence of ATP, and a radiolabeled tracker DNA substrate was added subsequently to the reaction mixtures. Throughout competitor DNA concentrations of 2.2-12.5 nM, greater inhibition of RECQ1 helicase activity on the tracker substrate was observed when the enzyme was preincubated with unlabeled forked duplex containing the translocating strand PTE lesion compared with the forked duplex containing PTE in the nontranslocating strand or neither strand (Fig. 2B). The greatest difference of approximately 3-fold was observed at 5.5 nM competitor DNA. These results suggest that during unwinding, RECQ1 becomes sequestered by the PTE lesion when the damage is on the translocating strand. In contrast, the ability of the forked duplex with the nontranslocating strand isopropyl phosphotriester linkage to sequester RECQ1 was markedly less and comparable to that of the undamaged fork duplex at all concentrations of competitor DNA tested.

Effect of PTE lesion on DNA unwinding catalyzed by the WRN and BLM helicases

To determine if the inhibitory effect of the PTE lesion was generalizable to other RecQ helicases which unwind with a 3' to 5' directionality, we tested purified recombinant Werner syndrome (WRN) and Bloom’s syndrome (BLM) helicases on the forked duplex DNA substrate series. A WRN protein titration demonstrated that a similarly reduced level of WRN helicase activity was observed for DNA substrates harboring the PTE lesion in either the translocating or nontranslocating strand compared to the control substrate (Fig. 3A and B). A maximal difference of approximately 6-fold was observed at 1 nM WRN; however, significant differences of 2- to 3-fold could be detected at WRN concentrations of 4 and 2 nM, respectively. The reduced effect at higher WRN protein concentrations was at least partially attributed to a plateau of control substrate unwound (~60%). From these results, we suggest that WRN is more sensitive to the lesion in the translocating strand. We next tested BLM for unwinding of the control and PTE forked duplex substrates. BLM helicase efficiently unwound the undamaged substrate and the substrate with the isopropyl PTE lesion in the top (nontranslocating) strand very similarly (Fig. 3C and D). However, the forked duplex harboring the PTE lesion in the translocating strand was poorly unwound by BLM at concentrations up to 2.5 nM. Less than 5% of the DNA substrate with the translocating strand PTE lesion was unwound by BLM (2.5 nM) compared to approximately 25% unwound for those substrates that contained the PTE in the nontranslocating strand or no PTE in either strand. Even at higher BLM concentrations, strand-specific inhibition was observed. These results suggest that BLM and WRN helicases behave differently in terms of their ability to tolerate a single isopropyl PTE lesion within the duplex when it resides in the nontranslocating strand.

E. coli SF1 UvrD and SF2 RecQ 3' to 5' helicases are differentially affected by the PTE lesion

As mentioned previously, the major contacts between SF1 helicases and DNA are with the bases; however, electrostatic interactions with the sugar phosphate backbone also exist. For SF2 helicases, the major contacts are thought to be with the phosphodiester backbone through ionic interactions with the helicase protein. To address potential differences between a SF1 helicase (UvrD) and SF2 helicase (RecQ) in a functional context when they encounter DNA damage, we compared their ability to unwind the PTE series of DNA substrates. E. coli RecQ helicase behaved similar to human RECQ1 and BLM helicases in which the helicase was more greatly inhibited by the PTE lesion residing in the translocating strand (Fig. 4A). Inhibition was significant as demonstrated by the 3-fold lower unwinding by RecQ on the PTE translocating strand substrate compared to the control substrate at RecQ concentrations of 0.3 nM and 0.6 nM. In contrast, throughout the UvrD protein
titration, very similar levels of unwinding were detected for all three DNA substrates (Fig. 4B). These results suggest that UvrD is highly insensitive to inhibition by the PTE lesion in either the nontranslocating or translocating strands.

SF2 Fe-S cluster DNA helicases FANCJ and ChlR1 are inhibited by the PTE lesion in a strand-specific manner

The preferential inhibition of SF2 RecQ helicases by a PTE lesion in the translocating strand raised the question if SF2 Fe-S cluster helicases (ChlR1, FANCJ) would behave similarly. In this case, the greatest inhibition would be observed by a PTE lesion in the top strand as opposed to the bottom strand since ChlR1 and FANCJ are 5′ to 3′ helicases whereas the RecQ helicases display a 3′ to 5′ directionality of translocation. Both FANCJ (Fig. 5A) and ChlR1 (Fig. 5B) were inhibited by the PTE lesion in a strand-specific manner, i.e., when the PTE lesion was positioned in the translocating strand. FANCJ (0.6 nM) was inhibited 3.2-fold (Fig. 5A). ChlR1 (0.12 nM) was inhibited 4.9-fold (5B). In contrast, little to no inhibition of DNA unwinding by either helicase was observed when the PTE lesion resided in the nontranslocating strand.

DNA unwinding by replicative helicases is unaffected by the PTE lesion in either the nontranslocating or translocating strands

Up to this point, we have largely focused our biochemical studies on DNA helicase involved in various aspects of the response to DNA damage or replication stress, or implicated in DNA repair pathways. Although there is some debate as to the assembly state for active unwinding catalyzed by these helicases, none of them are known to exist as hexameric ring-like structures, a configuration that is characteristic of the prokaryotic and eukaryotic replication fork helicases. To assess the impact of the PTE lesion on DNA unwinding catalyzed by helicases that operate at the replication fork to unwind parental duplex so that it can serve as a template for DNA synthesis, we tested E. coli DnaB and M. thermoautotrophicum MCM helicases for unwinding this series of DNA substrates. Since DnaB is a 5′ to 3′ helicase and MCM is a 3′ to 5′ helicase, we were interested in the possibility that these helicases would display opposite profiles for sensitivity to the PTE substrates. Results from helicase protein titrations demonstrated that neither MCM (Fig. 6A) nor DnaB (Fig. 6B) was affected by the PTE lesion in either the nontranslocating or translocating strand. These results suggest that replicative helicases, unlike SF2 DNA repair helicases, are fully able to tolerate the PTE lesion.

DISCUSSION

PTE adducts that arise from exposure to genotoxic chemicals represent a class of DNA lesions which are largely understudied. Although ample evidence exists for PTE-forming agents both in vitro and in vivo, in-depth molecular studies of their biochemical and biological consequences are lacking. In this study, we have examined the effect of a site-specific PTE on the ATP-dependent DNA unwinding reactions catalyzed by a panel of DNA helicases that are distinguished by 1) their amino acid conservation in the helicase domain; 2) their assembly state; 3) their mechanism of DNA unwinding; and 4) their biological functions. Several major findings were obtained from this study. Human SF2 DNA repair helicases (with the exception of WRN) are preferentially inhibited by a single PTE residing in the translocating strand, whereas a PTE in the nontranslocating strand exhibits more modest effects on helicase inhibition. For WRN, a PTE lesion in either the nontranslocating or translocating strand posed a formidable inhibition to DNA unwinding. Interestingly, RECQ1 was sequestered by the isopropyl PTE lesion residing in the translocating strand, consistent with the greater inhibition of helicase activity compared to the lesion in the nontranslocating strand. A SF1 helicase (UvrD) from E. coli was largely unaffected by the PTE positioned in either strand of the DNA substrate, whereas the SF2 E. coli RecQ helicase, like its human counterparts, was inhibited by the PTE lesion residing in the translocating strand. These results are consistent with evidence that SF1
and SF2 helicases unwind duplex DNA by distinct mechanisms. Neutralization of the sugar phosphate backbone or a steric effect imposed by the isopropyl ester linkage in the helicase translocating strand is apparently responsible for interference with the ability of SF2 helicases to efficiently complete unwinding of the DNA substrate. Here it should be emphasized that the PTE deters unwinding, but does not pose a complete block since appreciable amounts of the DNA substrate could be unwound, particularly at the higher protein concentrations. In contrast, the SF1 UvrD helicase, was quite resistant to inhibition by the PTE lesion, irrespective of the strand it is located. This latter observation was somewhat surprising in light of published data from the Yang lab on a series of crystal structures of UvrD complexed with DNA and ATP hydrolysis intermediates (13). The proposed wrench-and-inchworm mechanism for DNA unwinding one base pair at a time by UvrD suggests that UvrD makes substantial contacts with the DNA phosphate backbone on both strands during unwinding. Apparently, UvrD can tolerate at least one isopropyl substitution, whereas SF2 helicases are sensitive to a single isopropyl backbone lesion, particularly when it resides in the translocating strand. Although UvrD-catalyzed DNA unwinding was previously shown to be unaffected by a thymine glycol base damage (36), the helicase was strongly inhibited by a covalent protein-DNA complex in the translocating strand; however, no inhibition was observed when the lesion was placed in the nontranslocating strand (37), suggesting lesion-specific effects on DNA unwinding activity that may correlate to the bulky nature of the lesion.

A third major conclusion from this work is that ring-like replication fork helicases are largely unaffected by the PTE lesion. This finding is also consistent with other studies that demonstrate an unwinding mechanism of replicative ring-like helicases that is distinct from many DNA repair helicases which are proposed to function as monomers or dimers. Our own published work shows that from a kinetic standpoint, WRN helicase unwinds forked DNA duplex substrates (similar to the one used in this study) as a monomer (38). Thus the ring-like helicases MCM and DnaB that operate at the replication fork can apparently tolerate the isopropyl moiety and neutralization of the phosphodiester moiety’s negative charge whereas the SF2 helicases are sensitive, particularly when the PTE resides in the translocating strand. Recently, the Trakselis lab suggested a steric exclusion and wrapping model for the MCM helicase in which the 3’ single-stranded tail is encircled by the helicase whereas the unwound 5’ single-strand is wrapped around defined paths on the exterior surface of the helicase (39). Such a mode of action by the MCM helicase is evidently not affected by the existence of the isopropyl moiety attached to the backbone or the backbone neutralization by the PTE linkage.

WRN, on the other hand, is sensitive to the PTE lesion in either the translocating or nontranslocating strand. Previously, WRN helicase activity was shown to be inhibited in a strand-specific manner by a synthetic vinylphosphonate linkage positioned in the translocating strand; however, no inhibition was observed when the lesion was placed in the nontranslocating strand (40). Apparently, the presence of the isopropyl ester lesion in the nontranslocating strand sugar phosphate backbone poses an impediment to WRN helicase progression during unwinding that is not observed with the polyvinyl modification. In an earlier study, it was reported that the SF1 E. coli helicase PcrA was strongly inhibited by the vinylphosphonate linkage only when it was located in the helicase translocating strand (41). This is interesting in light of our observation that UvrD, which shares considerable sequence homology to PcrA, was largely unaffected by the isopropyl lesion in the sugar phosphate backbone of either the nontranslocating or translocating strand. The two SF1 helicases UvrD and PcrA may unwind double-stranded DNA by a fundamentally different mechanism, resulting in differential effects of DNA lesions. From a mechanistic standpoint, the ssDNA bases are better stacked and better ordered in the UvrD-DNA complex than in PcrA, which may compensate for the loss of backbone interactions in the UvrD case (13). Although there is some debate regarding the assembly
state of UvrD, kinetic evidence suggests that UvrD unwinds double-stranded DNA as a dimer (42) but can translocate along single-stranded DNA as a monomer (43), whereas PcrA behaves as a monomer to unwind duplex DNA (44). Thus, the distinct assembly states of UvrD and PcrA may play a role in how well the helicase tolerates a phosphate backbone modification during unwinding. *E. coli* RecQ is believed to unwind DNA as a monomer (45;46), suggesting a fundamentally different helicase mechanism compared to UvrD which may contribute to the differential effect of the isopropyl lesion on helicase activity by the two enzymes.

Assembly state of the eukaryotic RecQ helicases is of considerable interest for understanding their mechanism of DNA unwinding. RECQ1 is able to form dimers and higher order oligomers, and while dimer formation gives preferentiality to DNA unwinding, RECQ1 monomers are still active (47). Although electron microscopy studies of purified recombinant Bloom’s syndrome protein (BLM) showed that BLM form hexameric ring-like structures (48), biochemical evidence suggests that a catalytically active BLM helicase core domain fragment behaves as a monomer (49). BLM was inhibited by the PTE lesion in the translocating strand, suggesting that BLM does not behave like the ring-like helicasess MCM or DnaB on these substrates. Consistent with this result, BLM helicase activity on a forked duplex substrate is strongly blocked by a polyglycol linkage placed in the translocating strand within the duplex region (33). RECQ1 is also inhibited by a polyglycol linkage in a strand-specific manner, whereas MCM helicase activity is unaffected by the polyglycol linkage (our unpublished data). From these results, we infer that BLM, RECQ1, and the other RecQ helicasess are not likely to behave as a ring helicase to unwind simple forked duplex DNA substrates. Perhaps the oligomeric ring structure of BLM acts upon Holliday junctions, which would be analogous to the well characterized RuvAB complex implicated in Holliday junction branch migration (50). In this context, purified recombinant full-length WRN protein was shown by electron microscopy to bind Holliday Junction DNA as an oligomer (51).

The inhibitory effects of the PTE lesions on SF2 helicases determined in this study are of interest in light of previously published work that has focused on more standard DNA base lesions (23). For example, thymine glycol, which is known to induce a significant localized structural change placing the base extrahelical, inhibited the human RecQ helicases WRN, BLM, and RECQ1 all in a strand-specific manner, whereas other helicasess (DinG, DnaB, UvrD) were unaffected by thymine glycol in either strand (36). In contract to the Fe-S helicase DinG, FANCJ was sensitive to thymine glycol in either the translocating or nontranslocating strand (36). These results would suggest that even within the Fe-S family of helicasess, there are distinctive effects of base damage on DNA unwinding efficiency and that some differences in DNA unwinding mechanisms are likely to exist. Recently, DNA charge transport was implicated in the detection of a mismatch by Fe-S repair proteins including the XPD helicase (52), suggesting that redox potential of the Fe-S cluster may also be involved in the search for certain helix-distorting DNA base lesions.

The finding that replicative helicasess are entirely tolerant of the PTE is of interest with regard to DNA damage checkpoint signaling. PTEs were shown to inhibit the elongation of modified oligonucleotides by a number of DNA polymerases (1). For example, the presence of a single isopropyl PTE linkage reduced the rate of DNA polymerization catalyzed by human DNA polymerase alpha, T4 DNA polymerase, and Sequenase (modified version of T7 DNA polymerase) (53). Numerous studies have suggested a model in which DNA unwinding by the replicative helicase uncoupled from DNA synthesis which is slowed or halted by DNA damage is responsible for the creation of single-stranded DNA at the replication fork which is bound by RPA, an early signal in DNA damage checkpoint activation. Our *in vitro* studies would be consistent with a model in which the replicative helicase fully tolerates
the presence of the PTE lesion, creating unwound strands. A PTE lesion in the template strand would presumably deter chain elongation by the replicative polymerase, leading to the checkpoint signal. Further studies of the biological effects of PTE lesions on checkpoint signaling, mutagenesis, and DNA repair processes are warranted, given the evidence from this work and others that that alkylation of the sugar phosphate backbone is likely to have unique effects on DNA metabolizing proteins and perturb normal cellular DNA transactions.

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FOOTNOTES

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6The abbreviations used are: BLM, Bloom’s syndrome protein; FA, Fanconi anemia; FANCJ, Fanconia anemia Group J protein; Fe-S, Iron-Sulfur; ICL, Interstrand cross-link; Phosphotriester (PTE); SF, Superfamily; WRN, Werner syndrome protein; XP, Xeroderma pigmentosum

FIGURE LEGENDS

FIGURE 1. Inhibition of RECQ1 helicase activity by an alkyl triester lesion. Helicase reactions (20 μl) were performed by incubating the indicated RECQ1 concentrations with 0.5 nM forked duplex DNA containing an isopropyl PTE lesion in the top strand (nontranslocating), bottom strand (translocating), or neither strand at 37 °C for 15 min under standard helicase assay conditions as described under “Materials and Methods.” A, lane 1, no enzyme control; lanes 2–11, indicated concentrations of RECQ1; ▲, heat-denatured DNA substrate control. A phosphorimage of a typical gel from helicase assays with each DNA substrate is shown. B, quantitative analyses of RECQ1 helicase data are shown with standard deviations (SD) indicated by error bars. C, Kinetic experiments were performed using the forked duplex DNA substrate (0.5 nM) at 17.6 nM RECQ1 as described under “Experimental Procedures”. D, Quantitative analyses of the helicase data represent the mean of at least three independent experiments with SD indicated by error bars. E, Kinetic experiments were performed using the forked duplex DNA substrate 5 nM incubated with 17.6 nM RECQ1. The reaction is initiated by simultaneously adding ATP and 500 nM oligo-dT and stopped at the indicated times. F, Quantitative analyses of the helicase data represent the mean of at least three independent experiments with SD indicated by error bars. Filled square, control undamaged forked duplex substrate; Filled triangle, substrate with bottom strand isopropyl PTE; filled circle, substrate with top strand isopropyl PTE.

FIGURE 2. RECQ1 is preferentially sequestered by the isopropyl PTE positioned in the helicase translocating strand. A, Sequestration assays with 8.8 nM RECQ1 and the indicated concentrations (0–12.5 nM) of the specified forked duplex competitor DNA molecules were performed as described under “Experimental Procedures.” Approximately 90% of the tracker DNA substrate was unwound in the absence of competitor DNA. B, Quantitative analyses of the helicase data represent the mean of at least three independent experiments with SD indicated by error bars. Filled square, control undamaged forked duplex substrate; Filled triangle, substrate with bottom strand isopropyl PTE; filled circle, substrate with top strand isopropyl PTE.

FIGURE 3. Sensitivity of the WRN and BLM helicases to the alkyl triester modification. Helicase reactions (20 μl) were performed by incubating the indicated concentrations of WRN (A)
or BLM (C) with 0.5 nM forked duplex DNA containing an isopropyl PTE lesion in the top strand (nontranslocating), bottom strand (translocating), or neither strand at 37 °C for 15 min under standard helicase assay conditions as described under “Experimental Procedures.” B and D, quantitative analyses of helicase data for WRN and BLM, respectively, are shown. Filled square, control undamaged forked duplex substrate; Filled triangle, substrate with bottom strand isopropyl PTE; filled circle, substrate with top strand isopropyl PTE. Helicase data represent the mean of at least three independent experiments with SD indicated by error bars.

FIGURE 4. *E. coli* helicases RecQ and UvrD are differentially affected by the isopropyl PTE lesion. Helicase reactions were performed by incubating the indicated *E. coli* RecQ (A) or UvrD (B) concentrations with 0.5 nM forked duplex DNA containing an isopropyl PTE in the top strand (nontranslocating), bottom strand (translocating), or neither strand under standard helicase assay conditions as described under “Experimental Procedures.” Quantitative analyses of helicase data are shown. Filled square, control undamaged forked duplex substrate; Filled triangle, substrate with bottom strand isopropyl PTE; filled circle, substrate with top strand isopropyl PTE. Helicase data represent the mean of at least three independent experiments with SD indicated by error bars.

FIGURE 5. Strand-specific inhibition of FANCJ and ChlR1 helicases by the isopropyl PTE lesion. Helicase reactions (20 μl) were performed by incubating the indicated concentrations of the specified helicase (A, FANCJ; B, ChlR1) with 0.5 nM forked duplex DNA containing a alkyl triester damage in either the top, bottom or neither strand at the indicated temperature and reaction conditions for 15 min as described under “Experimental Procedures”. Quantitative analyses of helicase data are shown. Filled square, control undamaged forked duplex substrate; Filled triangle, substrate with bottom strand isopropyl PTE; filled circle, substrate with top strand isopropyl PTE. Helicase data represent the mean of at least three independent experiments with SD indicated by error bars.

FIGURE 6. The replicative helicases DnaB and MCM are insensitive to the isopropyl PTE lesion in either the translocating or nontranslocating strand. Helicase reactions (20 μl) were performed by incubating the indicated concentrations of the specified helicase (A, MCM; B, DnaB) with 0.5 nM forked duplex DNA containing an isopropyl PTE lesion in either the top, bottom or neither strand at the indicated temperature and reaction conditions as described under “Experimental Procedures.” Quantitative analyses of helicase data are shown. Filled square, control undamaged forked duplex substrate; Filled triangle, substrate with bottom strand isopropyl PTE; filled circle, substrate with top strand isopropyl PTE. Helicase data represent the mean of at least three independent experiments with SD indicated by error bars.
Isopropyl Phosphotriester Linkage

Scheme 1
Table 1. Oligonucleotide Substrates Used in This Study

| DNA substrate | Sequence (5′ to 3′) |
|---------------|---------------------|
| Control       | GACGCTGCCGAATTCTGGCTTGCTAGGACCAATTGGTCCTAGCAATGTAATCGTCTATGACGTC |
| PTE - B       | GACGCTGCCGAATTCTGGCTTGCTAGGACCAATTGGTCCTAGCAATGTAATCGTCTATGACGTC |
| PTE - C       | GACGCTGCCGAATTCTGGCTTGCTAGGACCAATTGGTCCTAGCAATGTAATCGTCTATGACGTC |

■ Denotes isopropyl PTE lesion

[Control: GACGCTGCCGAATTCTGGCTTGCTAGGACCAATTGGTCCTAGCAATGTAATCGTCTATGACGTC]

[PT-0-B: GACGCTGCCGAATTCTGGCTTGCTAGGACCAATTGGTCCTAGCAATGTAATCGTCTATGACGTC]

[PT-0-C: GACGCTGCCGAATTCTGGCTTGCTAGGACCAATTGGTCCTAGCAATGTAATCGTCTATGACGTC]
| Helicase | Organism       | Super Family | Assembly State<sup>a</sup> | Directionality | Function                                                                 | Mutant Phenotype<sup>a</sup> |
|----------|----------------|--------------|-----------------------------|----------------|---------------------------------------------------------------------------|-----------------------------|
| MCM      | *M. thermoau-totrophicum* | AAA<sup>+</sup> | hexamer                     | 3’→ 5’         | replication fork unwindning                                               | lethal                      |
| DnaB     | *E. coli*      | 4            | hexamer                     | 5’→ 3’         | replication fork unwindning                                               | lethal                      |
| UvrD     | *E. coli*      | 1            | monomer-translocase dimer-helicase<sup>b</sup> | 3’→ 5’         | nucleotide excision repair, mismatch repair, replication, recombination | elevated mutation frequency; UV sensitivity |
| RecQ     | *E. coli*      | 2            | monomer                     | 3’→ 5’         | recombination                                                             | recombination defects       |
| RECQ1    | *H. sapiens*   | 2            | monomer/dimer/multimer<sup>b</sup> | 3’→ 5’         | recombinational repair                                                    | elevated sister chromatid exchange |
| WRN      | *H. sapiens*   | 2            | monomer/multimer<sup>b</sup> | 3’→ 5’         | recombinational repair                                                    | Werner syndrome             |
| BLM      | *H. sapiens*   | 2            | monomer/multimer<sup>b</sup> | 3’→ 5’         | suppression of sister chromatid exchange                                  | Bloom’s Syndrome            |
| FANCJ    | *H. sapiens*   | 2            | ?                           | 5’→ 3’         | cross-link repair                                                         | Fanconi anemia              |
| ChlR1    | *H. sapiens*   | 2            | ?                           | 5’→ 3’         | suppression of sister chromatid cohesion defect                           | Warsaw Breakage syndrome    |

<sup>a</sup> See text for details and references

<sup>b</sup> Evidence for multiple assembly states
Fig. 1
Labeled DNA substrate (0.5 nM) (Control or PTE-B or PTE-C) + ATP

Initiate reaction with RECQ1 (17.6 nM)

37°C for indicated time

Quench and Analysis

---

Fig. 1
E

Labeled DNA substrate (5 nM) (Control or PTE-B or PTE-C) +
RECO1 (17.6 nM)

37°C, 3 min
Initiate reaction with ATP and dT120 (500 nM)

37°C for indicated time

Quench and Analysis

F

% Unwound

Time (sec)

Fig. 1
A

RECQ1 (8.7 nM) + ATP
Unlabeled competitor DNA (Control or PTE-B or PTE-C)

Preincubate 3 min, 37° C

Tracker substrate (0.5 nM)

7 min, 37° C

Quench and Analysis

B

Unwinding (% Control)

Competitor DNA (nM)

Control
PTE-B
PTE-C

Fig. 2
Fig. 3
Fig. 3

C

BLM (nM)

Control

PTE-B

PTE-C

D

% Unwound

BLM (nM)

Control

PTE-B

PTE-C

Fig. 3
Fig. 4
Fig. 5
Fig. 6
DNA repair and replication fork Helicases are differentially affected by an Alkyl phosphotriester lesion
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