RecQ helicases are believed to function in repairing replication forks stalled by DNA damage and may also play a role in the intra-S-phase checkpoint, which delays the replication of damaged DNA, thus permitting repair to occur. Since little is known regarding the effects of DNA damage on RecQ helicases, and because the replication and recombination defects in Werner syndrome cells may reflect abnormal processing of damaged DNA associated with the replication fork, we examined the effects of specific bulky, covalent adducts at N⁶ of deoxyadenosine (dA) or N² of deoxyguanosine (dG) on Werner (WRN) syndrome helicase activity. The adducts are derived from the optically active 7,8-diol 9,10-epoxide (DE) metabolites of the carcinogen benzo[a]pyrene (BaP). The results demonstrate that WRN helicase activity is inhibited in a strand-specific manner by BaP DE-dA adducts only when on the translocating strand. These adducts either occupy the minor groove without significant perturbation of DNA structure (trans acts) or cause base displacement at the adduct site (cis acts). In contrast, helicase activity is only mildly affected by intercalating BaP DE-dA adducts that locally perturb DNA double helical structure. This differs from our previous observation that intercalating da adducts derived from benzo[c]phenanthrene (BcPh) DEs inhibit WRN activity in a strand- and stereospecific manner. Partial unwinding of the DNA helix at BaP DE-dA adduct sites may make such adducted DNAs more susceptible to the action of helicase than DNA containing the corresponding BcPh DE-dA adducts, which cause little or no destabilization of duplex DNA. The single-stranded DNA binding protein RPA, an auxiliary factor for WRN helicase, enabled the DNA unwinding enzyme to overcome inhibition by either the trans-R or cis-R BaP DE-dG adduct, suggesting that WRN and RPA may function together to unwind duplex DNA harboring specific covalent adducts that otherwise block WRN helicase acting alone.

DNA damage evokes a cellular response by a genome surveillance system that senses DNA structural perturbation at the site of the lesion and elicits an appropriate response that may involve direct repair of the lesion, stabilization of the replication fork, or induction of apoptosis (1).

Inhibition of Werner Syndrome Helicase Activity by Benzo[a]pyrene Diol Epoxide Adducts Can Be Overcome by Replication Protein A*§

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Cellular pathways of DNA metabolism are influenced by DNA lesions, and since DNA unwinding enzymes known as helicases are among the first proteins to encounter DNA damage, it is of interest to understand how helicase action is modulated by interaction with chemically modified DNA. Although the mechanism for DNA unwinding has been studied for several helicases (2–4), only limited information is available regarding how specific covalently linked adducts affect helicase function. Of particular interest to us has been the Werner (WRN) helicase that is defective in the premature aging and genome instability disorder Werner syndrome (WS) (5). The WRN helicase belongs to the RecQ family of Superfamily 2 DNA helicases (5) and has been shown to unwind double-stranded DNA in a 3’ to 5’ direction with respect to the strand on which it is presumed to translocate (6). Biochemical characterization of the DNA substrate specificity of WRN helicase has revealed that the enzyme has the ability to target DNA replication/repair intermediate structures by its ability to recognize DNA junctions (7). Analysis of presteady state kinetics of WRN helicase indicated that the burst amplitude (unwound 19-base pair (bp) forked duplex molecules) was very close to the WRN protein concentration, suggesting that WRN can function as a monomer to unwind the duplex DNA substrate (8). WRN is a much more processive helicase in the presence of the human single-stranded DNA binding protein replication protein A (RPA), enabling the enzyme to unwind DNA duplexes as long as ~850 bp (6, 9). The biological significance of the WRN-RPA interaction is not well understood but is likely to relate to the implicated role of RPA in DNA replication, repair, or recombination.

The replication (10–13) and recombination (14–16) defects observed in WS cells may reflect abnormal processing of specific structures associated with the replication fork or a DNA recombination intermediate. WRN and other RecQ helicases have been proposed to function in repairing replication forks that have been stalled by DNA damage (17–19). In addition, RecQ helicases may play a role in the intra-S-phase checkpoint, which delays the replication of damaged DNA, thus permitting repair to occur (20). Studying the effects of DNA damage on the activity of RecQ helicases may lead to insight on how these helicases act upon encountering specific DNA adducts and how the absence of a given RecQ helicase contributes to genomic instability.

Benzo[a]pyrene (BaP) in the diet and in the air from combustion of fuel and tobacco is one of the most potent carcinogens to which humans are frequently exposed (21). A metabolic pathway involving cytochrome P450 and epoxide hydrolase converts BaP to BaP diol epoxides (BaP DEs) (22). Two diastereomers (each of which exists as a pair of enanti-
omers) of a given bay region DE are metabolically possible, one diaste-
reomer (DE-1) in which the benzylic hydroxyl group and the epoxide
oxygen are cis and one in which these two groups are trans (DE-2). Cis
or trans opening of the epoxide ring of BaP DE by the exocyclic amino
group \( N^6 \) or \( N^7 \), respectively, as shown. The partially saturated benzo-ring that is the
site of covalent attachment of the nucleoside is shown in boldface type. The absolute
configuration at C-10 is retained upon cis opening and inverted upon trans opening of
each DE.

The stereoisomeric BaP DE adducts at dG and dA provide a variety of
structural motifs within duplex DNA that have been well characterized
by NMR studies (24, 25). For cis-opened BaP DE-dG adducts, both 10R
and 10S enantiomers cause flipping out of both the modified base and its
complement from the DNA helix (26, 27). BaP DE-dG trans adducts
occupy the minor groove, with the hydrocarbon moiety of the 10R and
10S adducts oriented in opposite directions relative to the helix axis
(toward the 3’ and 5’ ends of the modified strand, respectively) and do
not significantly distort the double helix of B-form DNA (28–30). In
contrast, both cis (31) and trans (32–35) opened dA adducts intercalate
between base pairs such that the aromatic moiety of the 10R adducts
inserts on the 5’ side of the adducted base, whereas the aromatic moiety
of the 10S adducts inserts on the 3’ side. Based on the NMR structures,
intercalation of the hydrocarbon results in buckling and twisting of the
base pairs in the immediate vicinity of the adduct as well as local
unwinding and overall bending of the helix axis.

The BaP DE DNA adducts are partially resistant to cellular repair
processes (36–39) and thus are likely to be encountered by other DNA-
processing enzymes. Although DNA polymerases are potential targets
(40–46), the ability of polycyclic aromatic hydrocarbon-DE DNA
adducts to interact adversely with other enzymes such as topoisomer-
as (47, 48) may also contribute to their carcinogenic effects. In addi-
tion to these classes of DNA-metabolizing enzymes, helicases are likely
to be subject to the adverse effects of DNA lesions induced by environ-
mental chemicals.

In the current study, we observe that the effects of BaP DE adducts
with defined structural motifs on WRN helicase activity are specific to
the nature of the modified base as well as to the strand on which the
lesion is located. Thus, a single BaP DE-dG adduct with either cis or
trans, \( R \) or \( S \) stereochemistry inhibited WRN in a strand-specific man-
ner, whereas either a cis or trans, \( R \) or \( S \) BaP DE-dA adduct had little
effect. These results suggest that the effects of BaP DE adducts on WRN
helicase activity are related to the base to which the adduct is covalently
attached and dependent on the structure of the BaP DE DNA adduct,
such that WRN helicase activity is sensitive to BaP DE dG adducts,
which cause flipped out bases or occupy the minor groove. The single-
stranded DNA-binding protein RPA, which serves as an auxiliary factor
for the WRN helicase, enabled WRN to overcome the inhibition exerted
by either the cis or trans BaP DE-dG adduct, suggesting that WRN and
RPA may function together to unwind duplex DNA harboring specific
covalent DNA adducts that otherwise block WRN helicase acting alone.

MATERIALS AND METHODS

Proteins—Recombinant hexahistidine-tagged WRN protein was
overexpressed using a baculovirus/Sf9 insect system and purified as
described previously (19). Recombinant RECQ1 helicase was overex-
pressed in insect cells using a baculovirus encoding recombinant human
RECQ1 kindly provided by Dr. Alessandro Vindigni (International Cen-
tre for Genetic Engineering and Biotechnology) and purified as previ-
ously described (49). Purified recombinant UvrD helicase was kindly
provided by Dr. Steven Matson (University of North Carolina at Chapel
Hill). Purified human RPA containing all three subunits (RPA70,
RPA32, and RPA14) was graciously provided by Dr. Mark Kenny (Albert
Einstein Cancer Center).

Nucleotides, Oligonucleotides, and DNA Substrates—[\( \gamma \text{-}^{32}P \)]ATP was
from PerkinElmer Life Sciences. Unadducted oligonucleotides were
purchased from Lofstrand Technologies. Oligonucleotides containing
diastereomerically pure cis- and trans-opened BaP DE-dA or BaP
DE-dG adducts were synthesized using a semiautomated procedure, essen-
tially as described (50), with a manual step for coupling of the BaP
DE-dA (51, 52) or BaP DE-dG phosphoramidites (53) as their pure 10R
or 10S diastereomers. The synthesized oligonucleotides were purified by
reverse-phase high pressure liquid chromatography after removal of the
5’-protecting dimethoxytrityl group. Typically, each oligonucleotide
was chromatographed twice, utilizing two different columns success-
ively: 1) a Hamilton PRP-1 column (7 \( \mu \text{m}, 10 \times 250 \text{ mm} \)) at 25 °C
(retention times between 16 and 18 min for all oligonucleotides) and 2)
a Waters X Terra MS C18 column (2.5 \( \mu \text{m}, 10 \times 50 \text{ mm} \)) at 25 °C
(retention times between 10 and 13 min at 45 °C). Elution was at 3
ml/min with a gradient that increased the proportion of solvent B in A
from 0 to 35% over 20 min, where solvent A is 0.1 M (NH\(_4\))\(_2\)CO\(_3\) buffer
and B is a 1:1 mixture of A with acetonitrile, both adjusted to pH 7.0–
7.5. The absolute configurations of the adducts are known from the
diastereomerically pure reactant phosphoramidites (51–53). Single-
stranded oligonucleotides with BaP DE-dA or BaP DE-dG adducts are
shown in Table 1. The corresponding forked duplex substrates, pre-
pared as previously described (7), are shown in Fig. 1, A and B.

Helicase Assays—Helicase assay reaction mixtures (20 \( \mu \text{l} \)) contained
30 mM HEPES (pH 7.4), 5% glycerol, 40 mM KCl, 100 ng/\( \mu \text{l} \) bovine
serum albumin, 2 mM MgCl\(_2\), 2 mM ATP, 0.5 mM DNA substrate, and
the indicated concentrations of WRN, RECQ1, or UvrD. Reactions were
initiated with the addition of the indicated helicase and then incubated
at 37 °C for 15 min. Reactions were quenched with 20 \( \mu \text{l} \) of Stop buffer
(35 mM EDTA, 0.6% SDS, 25% glycerol, 0.04% bromphenol blue, 0.04% xylene cyanol) containing a 10-fold excess of unlabeled oligonucleotide
of the same sequence as the labeled strand of the DNA fork substrate.
The products of the helicase reactions were resolved on nondenaturing

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**WRN Helicase Inhibition by Benzopyrene Adducts**

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12% polyacrylamide gels. Radiolabeled DNA species in polyacrylamide gels were visualized using a PhosphorImager and quantitated using ImageQuant software (Amersham Biosciences). The percentage of helicase activity was calculated by the formula, percentage of helicase activity = 100 × (P/(P + S)), where P is the product and S is the residual substrate. The values of P and S have been corrected after subtracting background values in controls having no enzyme and heat-denatured substrate, respectively. Helicase data represent the mean of at least three independent experiments, with mean ± S.D. shown by error bars.

For helicase sequestration studies, WRN (3 nM in a 20-μl reaction) was preincubated with 0–500 fmol of the indicated unlabeled single-stranded DNA or unlabeled forked duplex DNA molecules in standard helicase reaction conditions as described above for 3 min at 37 °C. After the 3-min incubation, 10 fmol of radiolabeled forked duplex tracker substrate was added to the reaction mixture and incubated for 7 min at 37 °C. Reactions were quenched and resolved on native polyacrylamide gels as described above. Typically, 75–90% of the tracker helicase substrate was unwound in reactions lacking the competitor DNA molecule.

For kinetic helicase assays, 160-μl reaction mixtures containing the standard helicase reaction salts (see above), 0.5 nM duplex DNA substrate, 4.6 nM WRN in the presence or absence of 12 nM RPA were incubated for 0–4 min at 37 °C. 20-μl reaction volumes were removed and quenched as described above at the specified times. To correct for duplex destabilization by RPA, the background value for displaced strand (≤10%) in RPA alone reaction mixtures was subtracted from those values obtained for the WRN-RPA helicase reaction products. UvrD kinetic assays were conducted similar to the WRN kinetic assays except that UvrD (12 nM) was incubated with the substrate for 0–8 min at 37 °C.

### RESULTS

To evaluate the effects of DNA structural perturbations induced by adducts formed from DEs of the chemical carcinogen BaP on WRN helicase activity, we have tested a series of related forked duplex DNA...
substances with a covalently bonded site-specific BaP DE adduct positioned centrally in the 22-bp duplex region on one of the two DNA strands. The sequence chosen for placement of the adduct within the duplex is found in the coding sequence (exon 7) of the p53 gene (54). The site of the adducted base, BaP DE-dG or BaP DE-dA (Table 1), corresponded to p53 hotspot codons 248 and 249, respectively. The design of the DNA substrates used in this study enabled us to assess the effects of adduct stereochemistry, orientation, strand occupation, and nature of the DNA structural perturbation on WRN helicase activity.

For both the adducted (BaP DE-dA or BaP DE-dG) and unadducted forked duplex (22-bp) substrates, the intact oligonucleotide was released with minimal degradation (<2%) from the blunt end by WRN exonuclease as observed on native gels and confirmed on urea-denaturing gels (data not shown). Similar unwinding of the DNA substrates by an exonuclease-defective mutant WRN protein (WRN-E84A) was also observed (data not shown). These results are consistent with the previous observation that displacement of short (16- and 22-bp) duplex substrates by WRN helicase activity is more rapid than digestion by the WRN exonuclease activity (55).

**Effect of BaP DE-dA Adducts on WRN Helicase Activity**—We first tested substrates with a single BaP DE-dA adduct situated on the strand opposite to that on which WRN translocates (substrates 1–5; Fig. 1A) (Fig. 2A). For all of the five substrates tested, the percentage of duplex unwound depended on the concentration of WRN present in the reaction (Fig. 2A). The quantitative results from these assays demonstrated that WRN unwinding was not affected by the BaP DE-dA adducts residing in the nontranslocating strand, regardless of their stereochemistry.

We next tested substrates with a single BaP DE-dA adduct situated on the strand on which WRN translocates (substrates 6–10; Fig. 1A) (Fig. 2B). As before, the percentage of duplex unwound depended on the concentration of WRN present in the reaction (Fig. 2B). At the two highest concentrations of WRN tested, 2.3 and 4.6 nM, a statistically significant difference in WRN unwinding could be detected for certain substrates of this group. At 2.3 nM WRN, unwinding of the cis-R BaP DE-dA was reduced by ~1.5-fold compared with the unadducted substrate. At 4.6 nM WRN, unwinding of the cis-S and cis-R BaP-DE-dA-adducted substrates was reduced by ~1.7-fold. The trans S isomer showed slight inhibition at the highest concentration of WRN tested, although not as profound as the inhibition by the cis isomer. The trans-R configuration showed no inhibition at any level of WRN.

The possibility exists that when WRN helicase encounters the adduct, it may stall or become blocked. If this situation occurred, only 13 base pairs of the duplex would remain, which may melt spontaneously at 37 °C. Hence, the lack of extensive WRN helicase inhibition when translocating on the adducted strand of substrates 7–10 may not reflect helicase unwinding past the adduct. To reduce the possibility of spontaneous fraying of the partially unwound DNA substrate, WRN helicase reactions were repeated at a temperature of 30 °C, which is 6 °C below the theoretical T_m of the 13-bp duplex calculated from G/C content and salt concentration. Under these conditions, helicase activity by WRN (4.6 nM) showed little or no inhibition with substrates 7–9 and ~2-fold inhibition with substrate 10 (supplemental Fig. 1). Similar results were obtained using 2.3 nM WRN (data not shown). Extents of unwinding of the adducted substrates relative to control at 30 °C were comparable with those observed at 37 °C. This would suggest that duplex unwinding is due to the helicase action. Overall, these results indicate that for intercalated BaP-DE-dA adducts residing in the translocating strand, cis-opened adducts are slightly more inhibitory than trans-opened adducts. However, the extent of inhibition (<2-fold) was not that dramatic for any of the substrates with translocating strand BaP-DE-dA adducts, trans or cis. Available NMR data suggest that these two types of adducts, unlike cis- versus trans-opened dG adducts, produce qualitatively similar perturbations in the DNA structure. The observed inhibition of WRN helicase activity by the BaP DE-dA adducts was strand-specific, since neither class of BaP-DA adducts residing in the strand opposite to the one on which WRN translocates hindered WRN helicase activity.

**Effect of BaP DE-dG Adducts on WRN Helicase Activity**—We were next interested in observing the effects of BaP DE-dG adducts on WRN
helicase activity, since these adducts, depending on their stereochemistry, have markedly different effects on B-form double-stranded DNA structure compared with the BaP DE-dA adducts. We first tested helicase substrates with the single BaP DE-dG adduct positioned centrally in the duplex tract on the strand opposite to the one WRN translocates (substrates 11–15). For all of the five substrates tested, the percentage of substrate unwound was dependent on the WRN concentration (Fig. 3). Relative to the unadducted substrate 11, WRN unwinding was not affected by the BaP DE-dG adducts residing in the nontranslocating strand, regardless of its stereochemistry.

We next tested substrates with a single BaP DE-dG adduct situated on the strand on which WRN translocates (substrates 16–20). As before, the percentage of duplex unwound depended on the concentration of WRN present in the reaction (Fig. 4). Significant inhibition of WRN helicase activity by all four BaP DE-dG adducts tested was observed at WRN protein concentrations of 1.15, 2.3, and 4.6 nM WRN, where there was appreciable unwinding of the unadducted forked duplex substrate. The extent of helicase inhibition was not dependent on the stereochemistry of the specific adduct, since the percentage of substrate unwound at each WRN concentration was not significantly different for each of the adducted substrates. Clearly, all four BaP DE-dG adducts were able to potently block WRN unwinding, achieving a maximal level of ~6-fold inhibition at a WRN concentration of 2.3 nM, in which the unadducted substrate 16 was unwound 50% (Fig. 4B). WRN exonuclease activity at the blunt end of the unadducted or adducted substrates was minimal, as evidenced by the appearance of an intact released oligonucleotide on native gels (Fig. 4A) and confirmed by analysis of products on urea denaturing gels (data not shown). Taken together, the results indicate that WRN unwinding is inhibited in a strand-specific manner by BaP DE-dG adducts; however, the stereochemistry or orientation of the adduct did not have a significant effect on the extent of inhibition.

WRN Is Not Sequestered by the BaP DE-dG Modified DNA—Certain helicases have been found to be sequestered by DNA molecules harboring various types of covalent lesions (for a review, see Ref. 56). If sequestration of WRN occurs by the covalent BaP adduct, preincubation of WRN with the unlabeled single-stranded DNA or forked duplex DNA containing the adduct should trap the WRN helicase and prevent it from unwinding a radiolabeled, unadducted forked duplex tracker substrate. Since WRN is profoundly inhibited by the trans-R-dG adduct situated on the strand on which WRN translocates, we chose this substrate to examine for potential sequestration of the WRN helicase.

When WRN was preincubated with either the single-stranded unadducted oligonucleotide S or trans-R-dG adducted oligonucleotide U, little to no inhibition of WRN helicase activity on the tracker substrate was observed at any level of the oligonucleotide tested (Fig. 5, A and B). However, both the 16-unadducted and 18-trans-R forked duplex DNA inhibited unwinding of the tracker substrate (Fig. 5, C and D). The ability of forked duplex but not single-stranded DNA to inhibit WRN helicase activity on a tracker substrate is consistent with previous results of sequestration assays with DNA molecules of different sequences (57), indicating that WRN preferentially interacts with the forked duplex

FIGURE 4. Effects of BaP DE-dG adducts situated in the strand on which WRN translocates. Reaction conditions are the same as in Fig. 2. A, phosphor images of typical gels. Shown for each gel is a no enzyme control (NE) (lane 1), 0.575 nM WRN (lane 2), 1.15 nM WRN (lane 3), 2.3 nM WRN (lane 4), 4.6 nM WRN (lane 5), and heat-denatured DNA substrate control (lane 6). B, percentage unwinding.
compared with single-stranded DNA. As seen in Fig. 5E, since there was little to no difference in the extent of unwinding of the tracker substrate at each level of the adducted and unadducted substrates, this result suggests that WRN was not preferentially sequestered by the forked duplex molecule bearing the trans-R DE-dG adduct relative to unadducted DNA.

**Effects of BaP DE-dG Adducts on RECQ1 and UvrD Helicase Activities**—The inhibition of WRN helicase activity by the trans-S-BaP and trans-R-BaP dG adducts positioned in the strand on which WRN translocates raised the question of whether other 3'-5' DNA helicases would be similarly affected or whether the inhibition was unique to WRN. Therefore, we tested RECQ1 helicase, another human enzyme that is a member of the RecQ family (superfamily 2), and *Escherichia coli* UvrD, a member of Superfamily 1, on selected DNA substrates used for the WRN helicase characterization.

Throughout the RECQ1 concentration range (2.5–20 nM), the unwinding activity catalyzed by RECQ1 helicase was inhibited for either adducted substrate compared with the unadducted substrate (Fig. 6). In the presence of 5 nM RECQ1, 39% of the 16-unadducted substrate was unwound, compared with only 2.6 and 4.7% of the 17-trans-S and 18-trans-R substrates, respectively (Fig. 6). In the presence of 10 nM RECQ1, 77% of the 16-unadducted substrate was unwound by RECQ1, whereas only 10% 17-trans-S substrate and 20% trans-R substrate was unwound (Fig. 6). At a 2-fold higher concentration of RECQ1 (20 nM), unwinding of the 17-trans-S and 18-trans-R substrates was only slightly greater compared with the level of unwinding catalyzed by 10 nM RECQ1, indicating that increasing RECQ1 helicase concentration does not fully overcome the inhibition exerted by these adducts.

We next tested the ability of UvrD to unwind the 17-trans-S and 18-trans-R helicase substrates (Fig. 7A). As evidenced by the quantitative data, the -fold inhibition of UvrD helicase activity was dependent on
the concentration of UvrD enzyme. For example, at 0.38 nM UvrD, 31% of the 16-unadducted substrate was unwound compared with 5.5 and 8.5% of the 17-trans-S and 18-trans-R substrates, respectively (Fig. 7A). At 1.5 nM, UvrD unwound 22 and 40% of the 17-trans-S and 18-trans-R substrates, respectively, compared with 83% of the 16-unadducted substrate (Fig. 7A). However, as UvrD concentration was increased, progressively more of either adducted substrate was unwound. At 3 nM UvrD, 44 and 68% of the 17-trans-S and 18-trans-R substrates, respectively, were unwound. At 6 nM UvrD, 77 and 90% of the 17-trans-S and 18-trans-R substrates, respectively, were unwound. These results indicate that although UvrD is effectively inhibited by the trans-R or trans-S dG BaP adducts at low UvrD concentrations, the inhibition can be overcome by increasing the UvrD concentration. A kinetic analysis of DNA unwinding by 12 nM UvrD demonstrated similar rates of helicase activity for the 16-unadducted, 17-trans-S, and 18-trans-R substrates (Fig. 7B), indicating that inhibition of UvrD helicase activity by the trans-S or trans-R dG BaP adduct is not observed at higher UvrD concentrations.

The ability of UvrD to unwind the adducted substrates at higher enzyme concentrations may occur due to the enzyme binding to both the forked and blunt ends of the DNA substrate, since UvrD is known to unwind blunt duplexes at higher concentrations (57). To address this possibility, we tested UvrD unwinding of a 22-bp blunt duplex corresponding to the double-stranded region of substrate 16 under the conditions used for the UvrD helicase assays with the adducted substrates. The results from these experiments, shown in supplemental Fig. 2, demonstrate that UvrD is able to unwind greater percentages of the blunt duplex substrate with increasing UvrD concentrations. These results are compared with UvrD unwinding of 16-unadducted, 17-trans-S, and 18-trans-R substrates. This comparison suggests that the ability of UvrD to overcome the inhibition by these adducts is at least partially due to unwinding from the blunt end of these substrates.

The ability of UvrD to overcome the helicase inhibition by the BaP DE-dG adduct at higher UvrD concentrations raised the possibility that WRN might behave similarly. To address this, we tested a 2-fold higher concentration of WRN helicase, 9.2 nM, on unwinding of the 18-trans-R substrate. As shown in Fig. 7C, only a slight increase in unwinding of the adducted substrate, ~1.7-fold, was observed at 9.2 nM WRN compared with 4.6 nM WRN. However, the level of unwinding of the adducted substrate by WRN was still ~3.5-fold less than that of the unadducted substrate.

Effect of RPA on WRN-catalyzed Unwinding of BaP DE-dG-adducted Substrates—The human single-stranded DNA binding protein RPA physically and functionally interacts with WRN helicase, enabling the enzyme to unwind long DNA duplexes efficiently. The functional interaction between WRN helicase and RPA may also be important to enable the enzyme to unwind damaged DNA substrates. To address this possibility, we investigated the effect of RPA on WRN unwinding of the 18-trans-R- and 20-cis-R-adducted substrates by performing kinetic assays on these and the unadducted substrate in the presence or absence of RPA. The adducted substrates chosen were those that were most poorly unwound by WRN in the absence of RPA in the 15-min reaction (Fig. 4).

The kinetics of unwinding of the unadducted substrate by WRN were linear throughout the 4-min time course, yielding an initial rate of 1.5 bp/min DNA substrate unwound (Fig. 8A). The 18-trans-R and 20-cis-R substrates were unwound very inefficiently by WRN, resulting in only 0.17 bp/min and 0.37 bp/min DNA substrate unwound, respectively (Fig. 8B and C). This corresponded to a 9-fold and 4-fold reduction in rates for modified substrates 18-trans-R and 20-cis-R, respectively, compared with the unadducted substrate. In the presence of RPA (12 nM), WRN helicase activity was significantly increased on the unadducted substrate, yielding an initial rate of unwinding of 7.3 bp/min over the first 1 min of the time course (Fig. 8A). For the 18-trans-R substrate, the presence of RPA also significantly increased WRN helicase activity to a rate of 6.6 bp/min substrate unwound during the first min of the time course (Fig. 8B). A comparison of the results revealed that the rate
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The unique structural motifs presented by a series of DNA adducts of BaP DEs provided an opportunity to study the importance of WRN helicase contacts with the DNA double helix during the course of unwinding when it encounters specific types of DNA damage. Since the stereochemistry and base to which the BaP DE adduct is covalently bound dictate the nature of the helix perturbation, we were able to assess the effects of defined DNA structural motifs on helicase-catalyzed DNA unwinding. The substrates with a single BaP DE-dA adduct situated on the strand opposite to that on which WRN translocates did not inhibit WRN helicase activity. Interestingly, we observed only modest inhibition by the cis or trans BaP DE-dA adduct positioned in the helicase-translocating strand. Previously, we reported that duplex DNA unwinding catalyzed by the WRN helicase is inhibited by benzo[c]-phenanthrene (BcPh) DE-dA adducts in a strand-, orientation-, and stereoisomer-dependent manner (58). The aromatic portions of the 1R and 1S trans-opened BcPh DE N6-dA adducts used in that study intercalate between base pairs in duplex DNA with some local stretching of the double helix to accommodate the intercalated aromatic ring. The nonaromatic ring of the trans BcPh adduct is situated in the major groove, whereas the intercalated aromatic portion projects toward but not into the minor groove. The profound inhibition of unwinding by BcPh adducts provides evidence that a bulky, intercalating covalent adduct can have major consequences for WRN helicase function. However, the results from the current study suggest that for intercalating BaP DE-dA adducts, local double helix perturbations of buckling, twisting, or underwinding do not significantly deter the WRN helicase reaction. Thus the effect of a given bulky covalent DNA adduct on helicase function may depend on its chemical nature and how the adduct resides in the DNA double helix. Notably, BcPh DE-dA adducts have no effect on the thermal stability (Tm) of DNA duplexes (59), whereas BaP DE-dA adducts result in substantially lowered Tm values relative to the corresponding unadducted duplexes (60). This observation indicates that BcPh DE-dA adducts do not destabilize the DNA duplex, whereas the corresponding BaP adducts do so. Partial unwinding at the site of BaP DE-dA adducts could facilitate the action of helicase, thereby possibly overcoming any unfavorable steric effects of the bulk of the adducts.

In contrast to the results with the BaP DE-dA adducts, either cis or trans BaP DE-dG adducts positioned in the helicase-translocating strand exerted profound inhibition of WRN helicase activity, whereas the same adducts positioned in the opposite strand to the one on which WRN translocates did not inhibit WRN unwinding of the substrate. These results indicate that WRN helicase activity is strongly inhibited in a strand-specific manner by two conformationally very different types of adducts: cis BaP DE-dG adducts, which cause a flipped out base pair, and trans adduct, which resides in the minor groove. Similar to WRN, human RECQ1 helicase was profoundly inhibited by a BaP DE-dG adduct in a strand-specific manner, suggesting that RecQ helicases may be similarly affected by other types of DNA damage. A possible mechanism for the profound inhibition of WRN or RECQ helicase activity by the minor groove BaP DE-dG adduct is suggested by a recently identified conserved helix-turn-helix motif found in RecQ helicases that was shown to mediate minor groove binding in the human DNA repair protein O6-alkylguanine-DNA alkyltransferase (61).

consistent with the idea that WRN helicase activity is particularly susceptible to minor groove perturbation, we demonstrated previously that WRN-catalyzed DNA unwinding is potently inhibited by the minor...
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groove-binding drugs distamycin A and netropsin as compared with other DNA-binding drugs (62). The adverse effects of biologically relevant DNA modifications, either covalent or noncovalent, on the catalytic activities of WRN and other RecQ helicas may be relevant to the genetic damage and cell transformation induced by the adducts and/or the mechanism of action of chemotherapeutic drugs such as distamycin analogs that position themselves in the minor groove.

From the helicase protein titration studies, we observed that the inhibition of DNA unwinding by the minor groove adduct on the helicase-translocating strand was observed at all concentrations of WRN or RECQ1 helicase tested. Despite the effective inhibition of WRN helicase activity by a single BaP DE-dG adduct in the strand along which the helicase translocates, the adducted forked duplex molecule did not trap WRN to any greater extent than the unadducted molecule. Coupled together with the observation that WRN does not preferentially degrade by exonuclease digestion the poorly unwound adducted substrate, the results suggest that WRN dissociates as rapidly from the BaP-modified DNA molecule as the unadducted DNA molecule.

RPA, a single-stranded DNA binding protein that is implicated in the processes of DNA replication, repair, and recombination, was shown to specifically stimulate WRN helicase unwinding of long DNA duplexes. For the first time, we demonstrate that the presence of RPA can enable WRN to overcome the inhibition of DNA unwinding exerted by a single covalent base adduct residing in the translocating strand within the duplex. Understanding the biological significance of the WRN-RPA interaction in the replication stress response may be important to deciphering the DNA metabolic defects in WS. WS cells have a prolonged S phase (11), asymmetry of DNA replication fork progression (63), slower rate of repair associated with DNA damage induced in S-phase, reduced induction of RAD51 foci, and a higher level of strand breaks (64). The in vivo evidence implicating WRN in the recovery of DNA synthesis after replication arrest poses the question of whether WRN functions together with RPA in a critical step to resolve a key replication or recombinational intermediate that arises from fork stalling or collapse. RPA has been found to co-localize with WRN upon replication arrest (65) and DNA damage (66), suggesting that the two proteins may indeed collaborate to perform certain cellular functions. As a component of the replication stress response, RPA may serve to enable WRN helicase to overcome DNA-blocking base lesions introduced by exposure to exogenous DNA-damaging agents such as benzopyrene or DNA damage introduced by endogenous biochemical processes, such as oxidation. A role for WRN in conferring resistance to the lesions N³-methyladenine and O⁶-methylguanine was recently suggested by cellular studies (67); however, the importance of WRN helicase function remains to be shown. In addition to base modifications, RPA may also enable WRN to unwind DNA substrates with alterations to the sugar phosphate backbone, as recently suggested by in vitro WRN helicase studies with DNA substrates that contain synthetic vinylphosphonate modifications (68). By its partnership with RPA, WRN may be able to overcome helicase inhibition and ensure normal DNA transactions despite the presence of a potentially mutagenic lesion.

In the absence of functional WRN protein, the rate of replication fork elongation emanating from individual bidirectional origins is abnormal, leading to asymmetric fork progression (63). One reason for the observed slower rates of replication fork movement and asymmetry of replication fork progression in WS cells compared with normal cells may be the inability to replicate past lesions in the template or to restart a stalled replication fork (63). This rationale supports a role of WRN in unwinding double-stranded DNA to promote fork progression when it encounters a blocking lesion. If this model is true, our results would suggest that WRN functions with RPA to unwind past certain covalent adducts in the template strand. To generate RPA-coated single-stranded DNA necessary to activate the ATR-dependent DNA damage response pathway in response to genotoxic lesions, a mechanism of decoupling helicase and polymerase activities at a replication fork was very recently elucidated (69). The evidence that WS cells have a defect in replication fork propagation that is more pronounced at certain genomic sites compared with others (63) may be a consequence of the absence of an appropriate signal mediated by the WRN-RPA interaction at a stalled replication fork.

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