Coordinate Action of the Helicase and 3′ to 5′ Exonuclease of Werner Syndrome Protein*

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Werner syndrome is a human disorder characterized by premature aging, genomic instability, and abnormal telomere metabolism. The Werner syndrome protein (WRN) is the only known member of the RecQ DNA helicase family that contains a 3′ → 5′-exonuclease. However, it is not known whether both activities coordinate in a biological pathway. Here, we describe DNA structures, forked duplexes containing telomeric repeats, that are substrates for the simultaneous action of both WRN activities. We used these substrates to study the interactions between the WRN helicase and exonuclease on a single DNA molecule. WRN helicase unwinds at the forked end of the substrate, whereas the WRN exonuclease acts at the blunt end. Progression of the WRN exonuclease is inhibited by the action of WRN helicase converting duplex DNA to single strand DNA on forks of various duplex lengths. The WRN helicase and exonuclease act in concert to remove a DNA strand from a long forked duplex that is not completely unwound by the helicase. We analyzed the simultaneous action of WRN activities on the long forked duplex in the presence of the WRN protein partners, replication protein A (RPA), and the Ku70/80 heterodimer. RPA stimulated the WRN helicase, whereas Ku stimulated the WRN exonuclease. In the presence of both RPA and Ku, the WRN helicase activity dominated the exonuclease activity.

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‡ The abbreviations used are: WS, Werner syndrome; RPA, replication protein A; WRN, Werner protein; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; bp, base pair(s); nt, nucleotide(s).

Cases appear to play important biological roles, most probably relating to DNA metabolism.

Cells from patients with WS show premature replicative senescence and an extended S-phase compared with cells derived from normal individuals (14, 15). WS cells are also hypersensitive to selected DNA-damaging agents including 4-nitroquinoline-1-oxide (16) and exhibit increased genomic instability indicated by elevated levels of DNA deletions, translocations, and chromosomal breaks (17, 18). These observations and previous studies support a role for WRN in replication and/or recombination pathways (13, 19). Furthermore, WS cells display some defects in telomere metabolism, including increased rates of telomere shortening (20) and deficiencies in repair at telomeres (21). The expression of telomerase in WS cell lines prevented premature replicative senescence (22) and partially reversed the hypersensitivity to 4NQO (23). These studies suggest that WRN plays an important role in general DNA, and possibly telomere, metabolism by participating in DNA repair, replication, and/or recombination pathways.

The biochemical properties and substrate specificities of purified WRN protein have been investigated in vitro in order to gain insight into the biological role of WRN in vivo. WRN helicase unwinds DNA duplexes with a 3′ → 5′ directionality and requires the action of the WRN ATPase (19). WRN helicase unwinds several DNA structures including duplexes with a 3′ single-stranded tail (6), forked duplexes (24, 25), some tetraplexes (26), and triplexes (24) and promotes the translocation of Holliday junctions (27). Replication protein A (RPA) stimulates WRN helicase to unwind long M13 partial duplexes (28), whereas it appears to have no effect on the WRN exonuclease (29). RPA and WRN physically interact (28) and co-localize in cells arrested during S-phase with hydroxyurea (27). These findings suggest that the WRN helicase may function in a complex with RPA to resolve replication fork blocks.

The substrate specificities and enzymology of the WRN 3′ → 5′-exonuclease has also been characterized. WRN exonuclease initiates digestion from 3′-recessed termini, gaps, and nicks, but displays little or no activity on single-stranded DNA (ssDNA), blunt-ended duplexes, or 3′-protruding strands (6, 30). WRN exonuclease activity on a duplex blunt end is stimulated by the presence of ssDNA regions in the duplex (31). In addition, we have previously demonstrated that the WRN 3′ → 5′-exonuclease is stimulated by a physical and functional interaction with the Ku heterodimer of 68 and 83 kDa, whereas Ku does not affect WRN helicase unwinding (32). Evidence supports an important role for Ku in non-homologous DNA end joining and telomere maintenance (33), implicating the WRN exonuclease in either or both of these biological processes.

WRN is the only human RecQ helicase family member identified thus far that contains an additional 3′ → 5′-exonuclease.
Interaction between WRN Catalytic Activities

activity. The biological significance of the WRN exonuclease and helicase activities are not yet known, and it is not clear whether both WRN activities contribute to the same biological process in a coordinated manner. To date, in vitro enzymatic studies of WRN have focused on the exonuclease and helicase activities separately. Thus, the simultaneous action of the WRN exonuclease and helicase on a single substrate has not been investigated. To investigate how the WRN helicase and exonuclease might coordinate, we have examined WRN function on DNA-forked duplexes, which we found serve as substrates for both WRN activities under identical conditions in vitro. These forked duplexes were designed to contain telomeric repeats as a first step to study WRN function in telomere metabolism. We find that the WRN helicase is active at the forked end of the substrate, whereas WRN exonuclease is active at the blunt end of the duplex. The WRN activities appear to act in concert on long forked duplexes. We also have examined the coordinate action of the WRN helicase and exonuclease in the presence of its protein partners, Ku and RPA.

EXPERIMENTAL PROCEDURES

Proteins—The heterotrimer of human RPA (RPA70, RPA32, and RPA14) was a generous gift from Mark Kenny (Albert Einstein Cancer Center, Bronx, NY) and was purified as described previously (34). Recombinant human Ku70/86 heterodimer, containing a histidine tag and purified using a baculovirus/insect cell expression system (35), was kindly provided by Dale Ramsden (University of North Carolina, Chapel Hill, NC). T4 polynucleotide kinase was purchased from New England Biolabs.

Recombinant wild type and mutant WRN proteins were purified using a baculovirus/insect cell expression system and contained N-terminal histidine tags to aid in the purification. The baculovirus construct for expression of the K-WRN variant, containing a point mutation in the helicase domain (K577M), was kindly provided by Matthew Gray (University of Washington, Seattle, WA). The baculovirus construct for the expression of the X-WRN variant, containing a point mutation in the exonuclease domain (E94A), was kindly provided by Shurong Huang and Judy Campisi (University of California, Berkeley, CA). Protein production and purification was as described previously (8). Purification was achieved by a combination of DEAE-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ), Q-Sepharose (Amersham Pharmacia Biotech), and Ni-NTA (Qiagen) chromatography. Protein concentrations were determined by the Bio-Rad assay using bovine serum albumin as a standard.

DNA Substrates—Oligonucleotides used for substrate preparations were ordered from Midland Certified Reagents Co. (Midland, TX) and were purified by trityl-selective perfusion high pressure liquid chromatography by the manufacturer (Table I). Additional purification of 32P end-labeled oligonucleotides was performed as required using non-denaturing polyacrylamide gel electrophoresis and ethanol precipitation. Oligonucleotides were 5'-end-labeled with [γ-32P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase (New England Biolabs) according to the manufacturer. Labeled oligonucleotides were annealed to the complementary oligonucleotide in a 1:2 molar ratio by incubation in 50 mM LiCl at 95°C for 5 min followed by cooling to room temperature. LiCl was used to avoid tetraplex formation in the G-rich strand. The duplex region of the fork contained one, two, or four telomeric repeats (TTAGGG) followed by 10 bp of unique sequence, which was included to promote proper alignment of the repeat units in the duplex. Oligonucleotides 1 and 2 were annealed to form a 16-bp forked duplex, oligonucleotides 3 and 4 were annealed to form a 22-bp forked duplex, and oligonucleotides 5 and 6 were annealed to form a 34-bp forked duplex. Oligonucleotides 7 and 8 were annealed to form a duplex blunt at both ends, and oligonucleotides 6 and 7 were annealed to form the duplex with a 3'-single-stranded tail. In all cases the G-rich strand was 5'-end-labeled. An analysis of the substrates by native polyacrylamide gel electrophoresis confirmed the presence of a single species and the lack of alternate products formed by potential misalignment of the telomere repeats.

WRN Helicase and Exonuclease Reactions—Reactions (10–20 μl) were performed in standard reaction buffer (40 mM Tris-Cl, pH 8.0, 4 mM MgCl2, 5 mM dithiothreitol, 2 mM ATP, and 0.1 mg/ml bovine serum albumin) unless otherwise indicated. In all cases the DNA substrate concentration was 0.5 nM, expressed as substrate molecules. Protein concentrations were as indicated in the figure legends. Reactions were initiated by the addition of WRN and were incubated at 37°C for 15 min, unless otherwise indicated.

Analysis of Helicase Products—Reactions were terminated by the addition of 3x stop dye (50 mM EDTA, 40% glycerol, 0.9% SDS, 0.1% bromophenol blue, and 0.1% xylene cyanol) to a 1× final concentration, along with a 10× molar excess of unlabeled competitor oligonucleotide. The unlabeled competitor was identical to the labeled strand of the duplex and was added to prevent reannealing of the unwound ssDNA products. For experiments that included RPA and/or Ku70/80, terminated reactions were incubated with proteinase K (8.3 ng/μl final concentration) at 37°C for 30 min prior to gel electrophoresis. This process disrupted aggregations of protein and DNA complexes that would fail to enter the native gel. Products were run on 12% native polyacrylamide gels, visualized using a PhosphorImager and quantitated using ImageQuant software (Molecular Dynamics, Inc.). The percent of displaced product was quantitated as described previously (28) using the following formula: percent displacement = 100 *[P/(total DNA)] where P is the amount of displaced strand product. Background correction was carried out using control reactions that excluded the enzyme and included either native or heat-denatured substrate.

Analysis of Exonuclease Products—Reactions were terminated by an addition of equal volume formamide stop dye (80% formamide, 0.5× Tris borate, 0.1% bromophenol blue, and 0.1% xylene cyanol). Products were heat-denatured for 5 min at 95°C, run on 14% denaturing polyacrylamide gels, and visualized using a PhosphorImager. For kinetic analyses conducted on the 16-bp forked duplex, the amount of radioactivity in each product band was quantitated using ImageQuant software. The proportion of products shortened to each length was determined by dividing the amount of radioactivity in a given product band by the total radioactivity quantitated in the lane. The following formula was used to estimate the amount of nucleotides excised (fmoles) that each product band represented: proportion of product length x fmoles total DNA molecules (initial substrate length – product length xⁿ). All values were corrected for background in the 0-min lane. Values were summed to estimate total nucleotides excised/time point. The estimated rate of excision was determined by plotting total fmoles nucleotides excised versus time and by calculating the slope in the linear range. The rate was normalized for the total fmoles of WRN (calculated as a monomer) in the reaction.

RESULTS

Both WRN Helicase and Exonuclease Are Active on Forked Duplex Substrates—The forked substrates used in this study contained 15-mer ssDNA tails that formed a “fork” at one end of double-stranded DNA that was blunt at the other end. These substrates contained variable numbers of telomeric repeats in the duplex region. We investigated the substrate structural requirements for WRN activity on a forked duplex. Previous studies showed that WRN unwinding of linear duplex DNA is stimulated by ssDNA tails and requires at least a 3’-ssDNA tail or internal ssDNA region (25). Similarly, internal ssDNA regions within a duplex substrate stimulated WRN exonuclease

| Table I |
|---|
| Oligonucleotides used in substrate preparations |
| 1 |
| 2 |
| 3 |
| 4 |
| 5 |
| 6 |
| 7 |
| 8 |
activity at the blunt or 3'-recessed end of the substrate (31). However, the ability of ssDNA tails to stimulate WRN 3'→5'-exonuclease activity had not been previously demonstrated. Therefore, we tested whether a covalent 3'-ssDNA tail or exogenous ssDNA could stimulate WRN digestion of a blunt-ended duplex (Fig. 1). No WRN exonuclease activity was observed on the blunt-ended substrate, and exogenous single strand DNA failed to stimulate digestion (Fig. 1). However, a substrate containing a blunt end and a 3'-ssDNA tail at the other end was digested by WRN starting from the blunt end in a 3'→5' direction. In contrast, digestion was not observed at the blunt end of the substrate that had a 5'-ssDNA tail instead (data not shown). In summary, our results indicate that a covalently attached 3'-ssDNA tail is sufficient to stimulate WRN exonuclease digestion at a blunt end, suggesting that a forked duplex would also be a substrate for digestion.

To examine WRN catalytic activities simultaneously on the forked duplexes, substrates were incubated with increasing amounts of the WRN protein, and reaction products were analyzed on a native gel to display the helicase activity and on denaturing gels to visualize the products of the 3'→5'-exonuclease activity. Analysis on native gels indicated that maximal unwinding by the WRN helicase but was susceptible to digestion by the WRN exonuclease. An analysis of the reaction products on a denaturing gel showed a decrease in the labeled 49-mer and an increase in progressively shorter products as a function of WRN concentration (Fig. 3B). Prominent products corresponded to fragments 39, 32, and 26 nt long. In contrast, when the opposite strand was 5'-labeled and incubated with WRN, a ladder of digestion products was not observed (data not shown).

In summary, the extent of WRN exonuclease digestion was greater on a fork that had a longer duplex region (34 bp), which was not unwound by the WRN helicase. In contrast, the exonuclease activity was limited on forks with shorter duplex regions (16 and 22 bp) that were unwound by WRN.

**Is the WRN Helicase Active on the 34-bp Fork?**—In the experiment with the 34-bp forked duplex, we did not see the labeled 49-mer strand on the native gel that should have appeared if the substrate had been unwound. One explanation for this is that the helicase was inhibited during reactions with this substrate, although the WRN exonuclease was clearly active. If this had been true, partial duplex structures (i.e., partially digested labeled strands still hybridized to the unlabeled complement) should have been evident when the reactions were analyzed on a native gel. The prominent 39- and 32-mer products observed on the denaturing gel (Fig. 3B) would have formed partial duplexes of 24 and 17 bp, respectively, with one forked end and one end with a 5'-ssDNA tail. These products should have been stable under the reaction and electrophoresis conditions according to the previous experiments with the 16- and 22-bp forks (see Fig. 2A, no enzyme controls) and would have migrated more slowly than the 49-mer product. However, the labeled products at WRN concentrations of 1.9–15 nM migrated as single strand fragments below the 49-mer ssDNA marker on the native gel. This is shown more clearly in Fig. 3C, in which the products from a reaction with the 34-bp forked duplex and 7.5 nM WRN were electrophoresed with labeled markers that bracketed this size range. These data suggest the appearance of the 39- and 32-mer ssDNA products on the native gel resulted from WRN helicase unwinding (at the forked end) of the shortened duplexes formed by the action of the WRN exonuclease (initiated at the blunt end). Consistent with this finding, the 32- and 39-mer products were not observed when helicase activity was eliminated by using ATPγS (Fig. 3C, lane 6). Instead, a shorter fragment (21–26 bp in length according to denaturing gel analysis, Fig. 4A, third panel, lane 6) was apparent, most probably because of the WRN exonuclease degrading the substrate to unstable duplex lengths (6–11-bp duplex + 15-mer tail). Therefore, the WRN helicase and exonuclease appeared to function in concert to remove the labeled strand of the 34-bp forked duplex.

**Influence of WRN Helicase Unwinding on the Progression of WRN Exonuclease**—The above results indicate that the WRN exonuclease progression was greatest on the 34-bp forked du-
plex and least on the 16-bp forked duplex. In contrast, the WRN helicase efficiently unwound the 16- and 22-bp forked duplexes but did not unwind the full-length 34-bp forked duplex. Our data confirm previous reports that WRN helicase is dependent on duplex length (28), however, the influence of duplex length on the WRN exonuclease has not been previously reported. One explanation for our observations is that the activity of the WRN exonuclease on the different forked duplexes was modulated by the extent of WRN helicase unwinding. To determine the influence of the WRN catalyzed unwinding on the WRN exonuclease function, we measured digestion independently of unwinding activity. We used a WRN variant (K-WRN) that contained a mutation in the ATPase domain (K577M), which abolished helicase activity but not exonuclease activity (4, 28). K-WRN digested all the substrates more extensively than wild type (Fig. 4A, lanes designated K). With the wild type enzyme, the shortest detectable products were 29 nt long for the 16-bp forked duplex and 26 nt long for the 22- and 34-bp forked duplexes. Because the ssDNA tails for all forks were 15 nt, this indicated that digestion slowed considerably or stopped 14 bp (16-bp forked duplex) and 11 bp (22- and 34-bp forked duplexes) from the single strand/duplex junction (Fig. 4A). However, with the helicase mutant (K-WRN), the digestion pattern shifted such that the shortest product was 21 nt, indicating that the exonuclease slowed or stopped 6 bp from the single strand/duplex junction for all three forks (Fig. 4A). Furthermore, this pattern was reproduced with the wild type enzyme in reactions that either lacked ATP or contained ATPγS (Fig. 4A). Thus, on all three substrates, the presence of an active WRN helicase decreased the extent of WRN exonuclease digestion.

To confirm that the exonuclease activity detected in the above experiment is intrinsic to WRN and not due to a potential contaminant in the protein preparation, we repeated the experiment with a WRN variant (X-WRN). This enzyme contained a mutation (E84A) that inactivated the exonuclease activity (5, 36) while preserving the helicase activity. No exonuclease digestion products were observed when the substrates were incubated with the E84A variant (Fig. 4A, lanes designated X, and Fig. 4B). However, the X-WRN helicase unwinds the 16- and 22-bp forked duplex but not the 34-bp forked duplex, similar to wild type (Fig. 4B).

The WRN Helicase Unwinding Activity Is More Rapid Than WRN Exonuclease Activity on the 16-bp Fork—One implication of the results with the 16-bp forked duplex is that unwinding by the helicase was sufficiently rapid so as to preclude extensive digestion by the exonuclease activity. Previous studies showed that the WRN exonuclease has limited activity on ssDNA (30, 37). We confirmed this finding by incubation of the 5’ end-labeled ssDNA strand (31-mer from the 16-bp forked duplex) with WRN in the presence or absence of ATP or with K-WRN (oligonucleotide 1, Table I, Fig. 5A). An analysis of the products on a denaturing gel revealed substrate shortening by only 1–2 nt, whereas no digestion products were observed with the exonuclease-deficient X-WRN. Similar results were obtained us-
nuclease digestion. Furthermore, these data suggest that the WRN helicase influences the WRN exonuclease by altering the DNA structure, making it a poor substrate for the exonuclease (i.e. converting dsDNA to ssDNA). Thus, the rate of duplex displacement by the WRN helicase affects the extent to which the substrate is degraded by the WRN exonuclease.

**RPA Modulates the WRN Helicase and Exonuclease on Forked Duplexes**—Next, we tested how WRN protein partners would affect the coordinate action of the WRN helicase and exonuclease on the 34-bp fork. To our knowledge, the ability of RPA to stimulate WRN helicase so far has only been reported using partial duplex M13mp18 ssDNA substrates (7, 28). These substrates offer a large region of ssDNA (7180 nt) upon which RPA can load via the stable binding mode, which covers 30 nt (38). Because the forked substrates described above contain only 15-nt ssDNA strands, presumably RPA must load via the weaker binding mode, which occludes 8 nt (38). Therefore, we tested whether RPA could stimulate WRN helicase on the short-tailed fork substrate. RPA (34 nM, heterotrimer) alone promoted approximately 10 ± 2% displacement of the 34-bp forked duplex (Fig. 6A). However, the incubation of WRN together with increasing amounts of RPA resulted in a complete unwinding of the 34-bp forked duplex and release of the full-length 49-mer-labeled strand (Fig. 6A). RPA stimulated WRN helicase in a dose-dependent manner, and up to 94 ± 1.1% of the 49-mer was displaced at 34 nM RPA (Fig. 6A). However, an analysis of the ratio of RPA protein/RPA binding site revealed that RPA more effectively stimulated WRN unwinding of M13 partial duplex structures compared with forked substrates. Near maximal stimulation of WRN unwinding on the forked duplex was achieved at an RPA concentration of 8.5 nM (heterotrimer) (Fig. 6A). Whereas, the concentration of 8-nt ssDNA binding sites on the forked duplex was 1–2 nt in the reaction (taking into account the two 15-nt ssDNA tails and possible thermal breathing). Therefore, at maximal WRN stimulation, the ratio of RPA molecules to binding sites (8 nt) was 4:1–8:1 for the 34-bp forked duplex compared with a ratio of one RPA molecule/binding site (30 nt) as reported previously for a 69-nt M13 partial duplex (28). The difference in efficiency of RPA-stimulated WRN helicase for the two substrates is likely to reflect the higher affinity binding of RPA to the 30-nt site compared with 8 or 15 nt (38). Nevertheless, RPA is able to stimulate WRN helicase regardless of the initial loading and binding mode.

Next, we asked whether RPA influences the WRN exonuclease activity on the 34-bp forked duplex. On the native gel, digested products migrating below the displaced 49-mer were apparent at low molar ratios of RPA (trimer):WRN (monomer) but not at higher ratios (Fig. 6A). Products shorter than 49 nt are mostly probably resulted from WRN exonuclease digestion of the fork at the blunt end. An analysis of the products on a denaturing gel indicated that RPA inhibited WRN exonuclease in a dose-dependent manner with strong inhibition at a RPA: WRN molar ratio of one or higher (Fig. 6B).

We observed previously that RPA does not affect the WRN exonuclease on duplex DNA substrates with a recessed 3' terminus, a substrate that WRN helicase fails to unwind (29). Therefore, we questioned whether the RPA inhibition of WRN exonuclease on the forked duplex used in this study was dependent on WRN helicase activity. To address this issue, we examined the ability of RPA to inhibit the exonuclease activity of K-WRN, which lacks helicase activity. In contrast to wild type WRN, there was no dose-dependent inhibition of the K-WRN exonuclease activity (Fig. 6C). This observation suggests that RPA inhibited WRN exonuclease by stimulating the WRN helicase to unwind the duplex, thereby converting dsDNA to ssDNA, a poor substrate for the WRN exonuclease.

**FIG. 3.** WRN helicase and exonuclease act in concert on a 34-bp forked duplex to remove the labeled strand. WRN protein was incubated with the 34-bp forked duplex (0.5 nM) under the standard reaction conditions for 15 min at 37 °C. Reactions were terminated in the appropriate stop dye, and products were run on a 12% native polyacrylamide gel to analyze exonuclease activity (panel A) and on a 14% denaturing polyacrylamide gel to analyze helicase activity (panel B). Representative phosphorimagery scans are shown for reactions containing 0 (lane 1), 0.5, 1.0, 2.5, 7.5, and 15 nM WRN (lanes 5–9, panel A and lanes 2–5, panel B). Panel A also shows a lower titration of WRN as indicated (lanes 2–4), and a heat-denatured substrate control (A). Values in panel B indicate the product length. Panel C, markers for the native gel. Reactions included either 0 nM (lane 1) or 7.5 nM WRN with 2 mM ATP (lane 2) or 7.5 nM WRN with 2 mM ATP-γ-S (lane 6). Markers were treated identically to WRN reactions and include a 49-mer displaced strand (lane 3), 37-mer oligonucleotide (lane 4), and a 31-mer oligonucleotide (lane 5) as described in Table I. Values indicate position of the markers and estimated position of the WRN products in lane 2.
RPA Stimulates WRN Helicase and Inhibits WRN Exonuclease Even in the Presence of Ku—Both RPA and the Ku 70/80 heterodimer interact with the WRN protein, yet they modulate different WRN catalytic activities; RPA stimulates the WRN helicase, whereas Ku stimulates the WRN 3′→5′-exonuclease (32). Both RPA and Ku function in DNA repair pathways and likely form a complex with WRN in vivo. However, it is not known how the simultaneous presence of RPA and Ku would influence the WRN activities on a single substrate. Therefore, the WRN helicase and exonuclease activities were examined in the presence and absence of RPA and Ku using the 34-bp forked duplex. Helicase products were analyzed on a native gel (Fig. 7A), and exonuclease products were run on a denaturing gel (Fig. 7B). First, we determined the effect of Ku on WRN activity. Ku alone (8.5 nM, heterodimer) did not alter the substrate (Fig. 7B, lane 4), however, the incubation of Ku (8.5 nM) with WRN (7.5 nM, monomer) shifted the exonuclease products to shorter fragments (Fig. 7B, compare lane 2 with 5). Heat-inactivated Ku was unable to stimulate the WRN exonuclease (Fig. 7B, lanes 6). The influence of Ku on the WRN helicase activity could not be determined with the 34-bp forked duplex, because WRN does not completely unwind this substrate (Fig. 7A, lane 5). However, Ku did not significantly affect the WRN helicase activity with the 16-bp forked duplex using up to an 8-fold molar excess of Ku (data not shown), consistent with previous reports on M13 substrates (32). Next, we repeated the experiment with RPA and WRN. RPA (8.5 nM, heterotrimer) alone destabilized a limited amount of the 34-bp forked duplex, resulting in the displacement of 11±3% of the 49-mer-labeled strand (Fig. 7A, lane 7). RPA stimulated the WRN helicase to displace 86±3% of the 49-mer strand but completely inhibited the WRN exonuclease (Fig. 7, A and B, lane 8). Heat-inactivated RPA failed to inhibit the WRN exonuclease, and the 49-mer ssDNA helicase products were no longer apparent (Fig. 7, A and B, lane 9). Reactions, which contained equal molar quantities of WRN, RPA, and Ku, resulted in the displacement of 88±2% of the 49-mer strand and a lack of digestion products (Fig. 7, A and B, lane 10). Heat inactivation of RPA restored the exonuclease products (Fig. 7, A and B, lane 11), whereas heat inactivation of Ku had no effect (Fig. 7, A and B, lane 12). These results indicate that in the presence of WRN, RPA, and Ku, the WRN helicase unwinding of the forked duplex is the predominant activity.

**DISCUSSION**

In this study, we demonstrate that a forked duplex serves as a substrate for the simultaneous action of both the WRN 3′→5′-helicase and 3′→5′-exonuclease. We have exploited this observation to systematically examine the functional interaction between the two WRN catalytic activities. The progression of the WRN exonuclease was decreased on forked duplexes that were completely unwound by the WRN helicase (16- and 22-bp forks) compared with a fork that was not unwound (34-bp fork). Inactivation of the WRN helicase increased the extent of WRN exonuclease digestion on all three forks. Furthermore, studies with the 16-bp forked duplex revealed that strand displacement by WRN helicase was more rapid than substrate digestion by the WRN exonuclease. In addition, RPA stimulated the WRN helicase to unwind the 34-bp forked duplex, and the Ku70/80 heterodimer stimulated the WRN exonuclease on the
substrate. In the presence of WRN, RPA, and Ku70/80, the stimulated WRN helicase activity prevailed, such that no exonuclease activity was detected. The biological relevance of the forked substrates and the implications for regulation of WRN activities on these substrates in vivo are discussed below.

It was previously observed that ssDNA regions within a duplex, including a bubble, could regulate the WRN exonuclease by stimulating its activity at a blunt end (31). Here, we report that a single 3'/H11032-ssDNA tail was sufficient to stimulate the WRN exonuclease at a duplex blunt end. However, the ssDNA must be covalently attached to the substrate to promote digestion by the WRN exonuclease (Fig. 1). The mechanism of this stimulation is not well understood. One possibility is that ssDNA regions enhance the loading of WRN on the substrate. Indeed, WRN has a higher affinity for ssDNA than dsDNA (8), and the presence of ssDNA or secondary structure within a duplex stimulates WRN binding to the substrate (31). Therefore, WRN may initially load on the 3'/ssDNA tail of the fork and subsequently interact with the blunt end perhaps by bending the DNA molecule and/or by forming a multimeric structure that interacts at both ends. Stimulation of WRN exonuclease at blunt ends by ssDNA regions and by Ku 70/80, a factor in non-homologous end joining, suggest that WRN may participate in the repair of double strand breaks that occur proximal to replication forks.

Our data show that the rate of WRN helicase unwinding exceeds the rate of WRN exonuclease on the 16-bp forked duplex. A, analysis of WRN exonuclease on ssDNA. Reactions contained 0.5 nM 5' end-labeled oligonucleotide 1 (Table I) and 0 nM WRN (lane 1), 7.5 nM WRN (lane 2), 7.5 nM WRN without ATP (lane 3), 7.5 nM K-WRN (lane 4), or 7.5 nM X-WRN (lane 5) and incubated for 15 min at 37 °C in standard buffer. Products were run on a 14% denaturing polyacrylamide gel. B, analysis of WRN helicase on the 16-bp forked duplex. Reactions containing 0.5 nM substrate and 7.5 nM WRN in standard buffer were incubated at 37 °C. Aliquots were removed and added to native stop dye after 0, 0.5, 1, 2, 4, 6, 8, 10, and 15 min (lanes 2–7), and products were run on a 12% native polyacrylamide gel. C, analysis of WRN exonuclease on the 16-bp forked duplex with ATP. Reactions containing 0.5 nM substrate and 7.5 nM WRN in standard buffer were incubated at 37 °C. Aliquots were removed and added to native stop dye after 0, 0.5, 1, 2, 4, 6, 8, 10, and 15 min, and products were run on a 14% denaturing polyacrylamide gel. D, analysis of WRN exonuclease on the 16-bp forked duplex without ATP. Reactions were conducted and analyzed as in C, with the exception that ATP was omitted from the incubation. E, quantitation of exonuclease products. The plot of fmol nucleotides excised versus time is shown for WRN reactions containing 2 mM ATP (filled squares) or no ATP (filled circles). Values represent the mean and standard deviation from three independent reactions and were calculated as described under “Experimental Procedures.”
incided with a lack of exonuclease progression with time (Fig. 5, B and C). Furthermore, the digestion products that are observed in this experiment could be attributed, at least in part, to limited exonuclease activity on the displaced ssDNA strands. Our data show that the extent of exonuclease digestion is increased either by inactivating the WRN helicase (Fig.

**FIG. 6.** RPA modulates WRN helicase and exonuclease activity on the 34-bp forked duplex. WRN protein (7.5 nM) was incubated with the 34-bp forked duplex (0.5 nM) and increasing amounts of RPA as indicated for 15 min at 37 °C. A, effect of RPA on the WRN helicase. Products were run on a 12% native polyacrylamide gel, ▲, heat-denatured substrate control. The percent displacement of the labeled 49-mer strand (full-length) was calculated as described under “Experimental Procedures” and plotted against RPA concentration (nM). Values represent the mean, and error bars represent the standard deviation from five independent experiments. B, effect of RPA on the WRN exonuclease. RPA amounts were 1.2, 4.2, 8.5, and 17 nM (lanes 4–7, respectively). Products were run on a 14% denaturing polyacrylamide gel. C, effect of RPA on the K-WRN exonuclease. Reactions were conducted as for wild type, and RPA amounts were 1.2, 4.2, 8.4, and 17 nM (lanes 2–5, respectively). Products were run on a 14% denaturing polyacrylamide gel.

**FIG. 7.** WRN helicase activity dominates the exonuclease in the presence of RPA and Ku. Reactions contained various combinations of WRN protein (7.5 nM monomer), Ku70/80 (8.5 nM heterodimer), and/or RPA (8.5 nM heterotrimer) as indicated and were incubated with the 34-bp forked duplex (0.5 nM) for 15 min at 37 °C under the standard conditions. Products were run on either a 12% native gel (A) or a 14% denaturing gel (B). ▲, indicates that the enzyme was heat-inactivated before addition to the reaction, ▲, heat-denatured substrate control. Phosphorimager scans shown are representatives of three independent experiments.
5D) or by lengthening the duplex, such that it is not completely unwound by the helicase (34 bp, Fig. 3A). Therefore, factors that regulate the WRN helicase activity, such as duplex length, can also affect the simultaneous WRN exonuclease activity on the same substrate. This stresses the importance of examining both WRN catalytic activities when testing novel potential DNA substrates.

Independent Activity of WRN Exonuclease on Forked Duplexes—For reactions in which the helicase was inactivated (K-WRN, no ATP, or ATPγS), the shortest detectable products (21 nt) indicate that digestion slowed or stopped 6 bp from the ssDNA/dsDNA junction (Fig. 4A). However, the potential exists for continued digestion of shortened duplexes that thermally melt into ssDNA strands, because WRN can digest ssDNA 1–2 nt (Fig. 5A). Therefore, the WRN exonuclease digested the forked duplexes to a minimum length of 6–8 base pairs. Under the reaction and electrophoresis conditions used, we empirically determined that duplexes of 12 bp and less are not thermally stable (data not shown). These data suggest that WRN-substrate interactions stabilized the shortened duplexes, allowing the exonuclease to continue to digest the duplex to a minimum length of 6–8 bp. This analysis supports the notion that WRN interacts with the duplex ahead of (5′) to the site of excision and thus the duplex ahead of unwinding at the forked end. This interpretation is in accord with models, in which helicase enzymes make contacts with the duplex ahead of unwinding in addition to contacts with the single strand required for loading (39, 40).

Concerted Activity of WRN Helicase and Exonuclease on Forked Duplexes—One limitation of the helicase assay is that only the completely displaced products can be detected. Consequently, if the enzyme were to partially unwind the substrate and then dissociate, the unwound portion would rapidly reanneal. Indeed, previous analyses of the WRN helicase and exonuclease indicate that the enzyme exhibits low processivity (6, 28). In this study, the finding that WRN helicase unwinding affects the WRN exonuclease progression on the forked duplexes now offers a way to monitor helicase unwinding when complete strand displacement does not occur.

On the 22- and 34-bp forked duplexes, the WRN exonuclease did not proceed past 11 bp from the ssDNA/duplex junction (fork end), however, when the helicase was inactivated, digestion progressed further (Fig. 4A). The protection of additional base pairs in the presence of the helicase suggests that the helicase unwinds a minimum of 5–11 bp at the forked end. This takes into account that the enzyme protects a few base pairs in the absence of helicase unwinding (see above). As mentioned above, duplexes ≤12 bp are thermally unstable under the conditions used. Thus, minimal unwinding by WRN (11 bp) easily destabilizes the 22-bp forked duplex but does not destabilize the 34-bp forked duplex. This may explain why the WRN helicase can unwind the full-length 22-bp fork (Fig. 2A) but requires the shortening of the 34-bp duplex length by the exonuclease in order to displace the duplex substrate (Fig. 3C).

A model for the simultaneous action of WRN helicase and exonuclease on forked duplexes is shown in Fig. 8. The simplest scenario is that the enzyme loads at the ssDNA/duplex junction. On short duplexes, displacement is rapid, and minimal digestion occurs, whereas on longer forked duplexes, unwinding and digestion act in concert to decrease the length of the duplex (Fig. 8, B and C). If the duplex length is decreased to an unstable length in a single round of enzyme binding (Fig. 8C), the duplex will thermally melt upon enzyme dissociation. However, if the remaining duplex is stable upon enzyme dissociation, the unwound portion will reanneal, and additional rounds of enzyme binding, action, and dissociation are required to achieve duplex displacement (Fig. 8E).

To our knowledge, this study is the first report that RPA stimulates WRN unwinding of a short ssDNA-tailed substrate, forked duplex versus M13 partial duplexes. Because the loading site for RPA is small on these forks (15 nt), our observations further support the importance of the physical interaction between WRN and RPA. Our data also suggest that the WRN-RPA complex could potentially function at replication forks or other DNA-forked intermediates in vivo. The mechanism of RPA stimulation of WRN helicase is not fully understood. Maximal RPA stimulation of WRN helicase unwinding is achieved at a lower RPA molecules/RPA binding sites ratio on M13 partial duplexes compared with the forked duplex (4–8-fold). Therefore, the efficiency of RPA loading on the substrate may influence its ability to stimulate WRN helicase; high affinity 30-nt binding mode on M13 substrates versus the unstable 8-nt binding mode on the forked duplex.

A previous analysis on M13 substrates suggest that RPA does not alter the processivity of WRN helicase unwinding but does increase the unwinding rate (28). Results in this study with the forked substrates also suggest that RPA may increase the rate of strand displacement by WRN helicase. On the 34-bp forked duplex, the RPA inhibition of the WRN exonuclease most probably resulted from rapid conversion of dsDNA to ssDNA via stimulation of WRN helicase unwinding, because the helicase-deficient K-WRN exonuclease was not inhibited (Fig. 6). Indeed, RPA inhibited WRN exonuclease even in the presence of Ku, a protein that stimulates the exonuclease. Thus, the rate of the RPA-stimulated WRN helicase may exceed the rate of Ku-stimulated WRN exonuclease. Alternatively, RPA may bind WRN more tightly than Ku. Analyses of binding affinities and of reaction kinetics are necessary to distinguish between the two models. Furthermore, RPA also inhibited Ku stimulation of WRN exonuclease digestion of the unwound ssDNA strands (Fig. 7B), indicating that RPA may protect ssDNA from digestion by WRN. Therefore, RPA may play an important role in regulating both the WRN helicase and exonuclease activity on specific substrates in vivo.

The observations made in this study in vitro regarding WRN activity on forked duplexes have implications for WRN activity in vivo. The forked duplexes used here resemble DNA structures that occur as intermediates during in vivo processes, including replication, repair, and recombination. Our studies suggest that the WRN exonuclease and helicase may act in concert to remove long segments of DNA that cannot be unwound by the helicase alone (Fig. 8). It has been reported that WRN exonuclease is active at nicks and gaps (30), and that the presence of ssDNA regions stimulates digestion at nicks (31). In our model (Fig. 9), the exonuclease would excise nucleotides starting at the nick or gap, and the helicase would displace DNA starting from the fork at the opposite end. The length of the duplex and presence of RPA and/or Ku could regulate the extent of digestion. Furthermore, parts of the fork structures are inherent in a D-loop recombination intermediate (Fig. 9B). Another member of the RecQ family of DNA helicases, BLM helicase (the protein defective in Bloom's Syndrome), unwinds D-loop structures and has been proposed to function in preventing illegitimate recombination (41). WRN also has been shown to unwind recombination intermediates, namely a Holliday junction, and is also predicted to function in preventing illegitimate recombination events, especially those induced by replication fork arrest (27). Indeed, Werner Syndrome cells show a hyper-recombination phenotype and extended S-phase (19). Our results indicate that the WRN helicase and WRN exonuclease could potentially coordinate to remove recombination intermediates.
Very recent reports suggest that WRN may play a role in processing telomeric ends (13). WRN interacts functionally and physically with Ku (32), and Ku localizes to telomeres and functions in telomere maintenance (42). Evidence exists for the formation of secondary structures at telomeric ends, consisting of a large t-loop stabilized by a D-loop (43). Our results suggest that a WRN-RPA helicase complex could potentially function to resolve secondary structure at telomeric ends even in the presence of Ku to allow access to replication and/or repair factors. Evidence for a role of RPA in telomere maintenance was recently obtained from studies in yeast strains containing raf1 mutations, the homologue of RPA p70 (44). Furthermore, WRN was observed to co-localize with telomere-binding factors in immortalized telomerase-independent human cell lines (45). WRN may either participate in this telomerase-independent pathway for lengthening telomeres or may facilitate this process by resolving secondary structure at telomeric ends.

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Coordinate Action of the Helicase and 3’ to 5’ Exonuclease of Werner Syndrome Protein
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