Human Werner Syndrome DNA Helicase Unwinds Tetrahelical Structures of the Fragile X Syndrome Repeat Sequence d(CGG)$_n$*

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Formation of hairpin and tetrahelical structures by a d(CGG) trinucleotide repeat sequence is thought to cause expansion of this sequence and to engender fragile X syndrome. Here we show that human Werner syndrome DNA helicase (WRN), a member of the RecQ family of helicases, efficiently unwinds G$^2$ bimolecular tetraplex structures of d(CGG)$_7$. Unwinding of d(CGG)$_7$ by WRN requires hydrolyzable ATP and Mg$^{2+}$ and is proportional to the amount of added helicase and to the time of incubation. The efficiencies of unwinding of G$^2$ d(CGG)$_7$ tetraplex with 7 nucleotide-long single-stranded tails at their 3’ or 5’ ends are, respectively, 3.5- and 2-fold greater than that of double-stranded DNA. By contrast, WRN is unable to unwind a blunt-ended d(CGG)$_7$ tetraplex, bimolecular tetraplex structures of a telomeric sequence 5’-d(TAGACATG(TTAGGG)$_2$TTA)-3’, or tetramolecular quadruplex structures of an Ig$\varepsilon$ switch region sequence 5’-d(TACAGGGGAGCTGGGGTAGA)-3’. The ability of WRN to selectively unwind specific tetrahelices may reflect a specific role of this helicase in DNA metabolism.

DNA helicases of the RecQ family unwind DNA with a 3’→5’ directionality and require ATP and Mg$^{2+}$ for catalysis. All members of the RecQ family of proteins share seven sequence motifs common to helicases, including the characteristic DexI box (1). Prokaryotic and yeast helicases of this family include Escherichia coli RecQ, Saccharomyces cerevisiae Sgs-1p, and Schizosaccharomyces pombe Rqh-1p. Three RecQ homologues have been identified in human cells: BLM, a helicase that is mutated in cells of Bloom’s syndrome patients (2); WRN,1 a helicase mutated in Werner syndrome (3); and RecQL, a helicase of unknown function (4). The in vivo functions of most helicases of the RecQ family are not fully understood. It appears, however, that these enzymes take part in diverse DNA transactions such as replication, repair, and recombination. E. coli RecQ is believed to initiate homologous recombination and to suppress illegitimate recombination (5, 6). RecQ is also thought to be involved in the reassembly of replication forks after their disruption by UV irradiation (7, 8).

1 The abbreviations used are: WRN, human Werner syndrome DNA helicase; G$^2$ DNA, bimolecular tetraplex DNA; G4 DNA, tetramolecular tetraplex DNA.

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A Werner Syndrome DNA Helicase Unwinds Tetraplex DNA

**RESULTS**

The trinucleotide repeat sequence d(CGG)ₙ has been shown to readily fold into hairpin structures (31–34) and to assemble into tetrahelical formations (25–27). Tetraplex structures of d(CGG)ₙ are bonded by guanine-guanine non-Watson-Crick hydrogen bonds and are stabilized by monovalent alkali cations (25–27). In investigating the unwinding of d(CGG)ₙ tetraplex structures by WRN, we first characterized these tetraplexes by defining requirements for their formation and determining their stoichiometry. As seen in Fig. 1A, 32P-5'-labeled d(CGG)ₙ incubated at 54 °C in the presence of Na⁺ yielded an electrophoretically retarded form whose amount increased exponentially as a function of the oligomer concentration. The second order kinetics of formation of the slowly migrating form of d(CGG)ₙ, as indicated by the linearity of a log/log plot of results presented in Fig. 1A (not shown), suggests that it was a multi-molecular complex. To determine the stoichiometry of this structure, 32P-5'-labeled d(CGG)ₙ, 3'-tail d(CGG)ₙ, or an equimolar mixture thereof was incubated at 54 °C in the presence of 300 mM NaCl. Slowly migrating structures of the oligomers were resolved from remaining single strands by nondenaturing gel electrophoresis. As seen in Fig. 1B, an equimolar mixture of the two oligomers yielded a third hybrid species in addition to the electrophoretically retarded forms of d(CGG)ₙ and 3'-tail d(CGG)ₙ.
FIG. 1. Formation of tetrahelical d(CGG)$_7$ complexes and their stoichiometry. A, second order formation of a slowly migrating d(CGG)$_7$ tetraplex complex. A constant amount of $^{32}$P-$5'-$labeled 3'-tail d(CGG)$_7$ at 0.01 mM was mixed with 0.03–3.5 mM of unlabeled 3'-tail d(CGG)$_7$ in a final volume of 10 μl of TE buffer, 300 mM NaCl. The DNA was denatured at 90 °C for 5 min, the mixtures were incubated at 54 °C for 21 h, and the reaction was terminated by the addition of 50 μl of ice-cold 60 mM KCl. Single-stranded and tetraplex 3'-tail d(CGG)$_7$ were resolved by electrophoresis at 4 °C through a nondenaturating 12% polyacrylamide gel containing 50 mM NaCl and 50 mM KCl in 0.5 × TBE buffer. The curve shows results of phosphorimager quantification of the accumulation of the electrophoretically retarded complex as a function of concentration of $^{32}$P-$5'-$labeled 3'-tail d(CGG)$_7$. An autoradiogram of the gel is shown in the inset. B, stoichiometry of the d(CGG)$_7$ tetraplex complex and requirements for its formation. Mixtures that contained 4.7 μM of $^{32}$P-$5'-$labeled d(CGG)$_7$, or 3'-tail d(CGG)$_7$, or a 1:1 mixture thereof in 10 μl of TE buffer, 300 mM NaCl were incubated at 54 °C for 21 h, and DNA single strands were resolved from their slowly migrating complexes by electrophoresis as in A above. Typical electrophoretic migration of the DNA oligomers and their complexes is shown in lanes 1–3. Requirements for complex formation: mixtures containing 4.7 μM of $^{32}$P-$5'-$labeled d(CGG)$_7$, 3'-tail d(CGG)$_7$, or 5'-tail d(CGG)$_7$, were incubated at 54 °C for 21 h in TE buffer with no salt. Nondenaturing gel electrophoresis resolution of the three DNA preparations is shown in lanes 4–6. Increasing amounts of d(CII)$_9$, were incubated at 54 °C for 21 h in TE buffer containing 300 mM NaCl. Electrophoretic resolution of the d(CII)$_9$ samples is shown in lanes 7–11. C, scheme of d(CGG)$_7$ tetraplex structures. Based on the findings in A and B above and on previously described results (25, 26), the bimolecular tetrahelices are depicted as dimers of two hairpins bonded by guanine quartets. Shown are G'2 bimolecular tetraplexes without or with a non-d(CGG) single-stranded tail at their 3' or 5' ends (dashed lines). Only two pairs of stacked guanine quartets are drawn in each tetraplex. The two hairpins are aligned against each other in one of several possible orientations (26).

d(CGG)$_7$. Similar results were obtained when d(CGG)$_7$ was incubated together with 5'-tail d(CGG)$_7$ (data not shown). The presence of three retarded bands in the mixtures of d(CGG)$_7$ and 5' or 3'-tail d(CGG)$_7$, indicated that the slowly migrating complexes were G'2 bimolecular structures. Additional results presented in Fig. 1B show that negligible amounts of G'2 structures of d(CGG)$_7$, 3'-tail d(CGG)$_7$, or 5'-tail d(CGG)$_7$, were generated in the absence of Na$^+$. To assess the role of hydrogen bonding in the formation of the complexes, guanine residues in d(CGG)$_7$ were substituted by inosines that lack a C2 amino group necessary for the formation of both Watson-Crick and non-Watson-Crick hydrogen bonds. As seen in Fig. 1B, no complex was generated by d(CII)$_9$ when increasing amounts of the oligomer were incubated in the presence of Na$^+$. This finding, as well as our previously reported observation that the d(CGG)$_7$ complex resisted methylation by dimethyl sulfate (25), suggests that the slowly migrating form of d(CGG)$_7$ was a bimolecular tetraplex complex stabilized by non-Watson-Crick hydrogen bonds (Fig. 1C).

Formation of electrophoretically retarded complexes of the telomeric sequences TeR2 and 5'-tail TeR2 and of the IgG switch region sequence oligomers Q and 5'-tail Q was also found to be cation- and DNA concentration-dependent (results not shown). The presence of three electrophoretically retarded bands of methylation-protected structures in a mixture of TeR2 and 5'-tail TeR2 oligomers indicated a bimolecular stoichiometry of these complexes (data not shown). Generation of five slowly migrating dimethyl sulfate-resistant species in a mixture of oligomer Q and 5'-tail Q suggested a tetramolecular stoichiometry of these G4 complexes (results not presented).

WRN Unwinds Tetraplex G'2 5'-Tail d(CGG)$_7$—Werner syndrome DNA helicase (WRN) incubated at 37 °C for increasing periods of time in a helicase reaction mixture progressively unwound $^{32}$P-$5'-$labeled G'2 5'-tail d(CGG)$_7$ (Fig. 2A). The
WRN-catalyzed unwinding reaction depended on the presence of Mg\(^{2+}\) and ATP. Further, ATP could not be substituted by its nonhydrolyzable analog γ-S-ATP (Fig. 2B). Similar results were obtained for the unwinding by WRN of G'2 3'-tail d(CGG)\(_7\) (not shown).

**WRN Requires a Single-stranded Tail for the Unwinding of Tetraplex d(CGG)\(_7\).**—To study the DNA substrate specificity of WRN, increasing amounts of the enzyme were incubated under standard helicase assay conditions with 300 fmol each of \(^{32}\)P-labeled 20-mer/46-mer partial duplex DNA or G'2 tetraplex forms of d(CGG)\(_7\), 3'-tail d(CGG)\(_7\), or 5'-tail d(CGG)\(_7\). As seen in Fig. 3, WRN resolved G'2 structures of 5'-tail d(CGG)\(_7\) and 3'-tail d(CGG)\(_7\) at efficiencies that were similar to or greater than that of the unwinding of a 20-mer/46-mer partial DNA duplex. Interestingly, under these conditions, WRN did not measurably unwind a partial double strand of hook d(CGG)\(_7\)-hook d(CGG)\(_7\) probably because of its high stability (results not presented). Thus, it appeared that WRN does not preferentially unwind all d(CGG)\(_7\)-containing DNA structures. Notably, WRN failed to measurably unwind G'2 d(CGG)\(_7\) that lacked a single-stranded tail (Fig. 3). Unwinding of G'2 d(CGG)\(_7\) could not be detected even in the presence of a 1.5-fold molar excess of WRN over this blunt-ended tetraplex (data not shown).

**WRN Unwinds Tailed G'2 d(CGG)\(_7\) More Efficiently than a Partial DNA Duplex.—**To assess the efficacy of unwinding by WRN of G'2 3'-tail d(CGG)\(_7\) relative to a 20-mer/46-mer DNA partial duplex, increasing amounts of WRN helicase were added to 300 fmol of either labeled DNA substrate, and proportions of displaced single strands were quantified by phosphorimager analysis. Average results of multiple experiments presented in Fig. 4 indicated that WRN unwind G'2 3'-tail d(CGG)\(_7\) at an efficiency that was ~3.5-fold higher than for partial DNA duplex. Whereas 50% of the G'2 3'-tail d(CGG)\(_7\) tetraplex became unwound in the presence of 9 fmol WRN, the unwinding of 50% of the 20-mer/46-mer partial duplex required 32 fmol of the helicase (Fig. 4). Similar analysis revealed that G'2 5'-tail d(CGG)\(_7\) was resolved by WRN at a ~2-fold greater efficiency than partial duplex DNA (results not shown).

**WRN Fails to Unwind Tetraplex Forms of Telomeric DNA and an IgG Switch Sequence.—**The ability of WRN to unwind tetraplex forms of guanine-rich sequences other than d(CGG)\(_7\) was examined. Increasing amounts of the helicase were incu-
bated with 300 fmol each of blunt-ended or 5' -tailed G'2 bimo-
molecular tetraplex forms of the telomeric sequence TeR2 or with
300 fmol of blunt-ended or 5' -tailed G4 four-molecular tetra-
plex forms of the IgG switch sequence Q. As seen in Fig. 5, no
displacement of single strands from any of the quadruplex
DNA structures was detected even at a molar excess of WRN
over tetraplex DNA substrate. Control partial DNA duplex was
completely unwound by WRN under the same reaction condi-
tions (not shown). As also seen in Fig. 5, amounts of tetraplex
TeR2, 5' -tail TeR2 and oligomer Q were diminished in the
presence of maximum amounts of the helicase. This decrease
was due to digestion of the DNA by the 3' → 5' WRN-associated
exonuclease, as demonstrated by visualizing DNA degradation
products by denaturing gel electrophoresis of the DNA (results
not shown). Hence, unlike 3' - or 5' -tailed G'2 d(CGG)₇, quad-
ruplex forms of telomeric DNA or of the IgG switch region
sequence could not be resolved by WRN.

DISCUSSION

Results presented in this paper demonstrate WRN helicase
is capable of unwinding G'2 bimolecular tetraplex structures
of the d(CGG) repeat sequence. Unwinding of G'2 d(CGG)₇ by
WRN required hydrolyzable ATP and Mg²⁺, and the extent of
the reaction was dependent on the amount of enzyme added
and time of incubation (Fig. 2). WRN required short single-
stranded tracts at the 3' or 5' ends of the G'2 d(CGG)₇ tetraplex
and a blunt-ended bimolecular d(CGG)₇ tetraplex could not be
unwound (Fig. 3). Unwinding by WRN of 3' - or 5' -tailed G'2
d(CGG)₇ was 3.5- and 2-fold, respectively, more efficient than
the unwinding of partial DNA duplex (Fig. 4). It is notable that
although double-stranded DNA is unwound by WRN at a 3' →
5' direction (3), G'2 tetraplex structures of d(CGG)₉ with both
3' and 5' single-stranded tails served as efficient substrates for
this helicase. It might thus be that the directionality of disrup-

![Fig. 4. Relative efficiency of unwinding of double-stranded DNA and tetraplex 3'-tail d(CGG)₇ by WRN. Increasing amounts of WRN (3.1–62.5 fmol) were added to DNA helicase reaction mixtures each containing 300 fmol of either ³²P-5'-labeled 20-mer/46-mer partial DNA duplex or ³²P-5'-labeled G'2 3'-tail d(CGG)₇. Following incubation at 37 °C for 15 min and termination of the reaction, displaced single strands were resolved from tetraplex 3'-tail d(CGG)₇ by nondenaturing gel electrophoresis as described in the legend to Fig. 2. Amounts of single-stranded and G'2 tetraplex 3'-tail (CGG)₇ were quantified by phosphorimager analysis. Results presented are the averages of four independent experiments.](image)

![Fig. 5. WRN helicase fails to unwind tetraplex forms of TeR2 DNA and oligomer Q. The indicated amounts of WRN protein were added to helicase reaction mixtures each containing 300 fmol of ³²P-5'-labeled bimolecular G'2 tetraplex forms of TeR2 or 5'-tail TeR2 or four-molecular G4 forms of oligomer Q or 5'-tail oligomer Q (see "Experimental Procedures"). Reaction mixtures without WRN were incubated at 100 or 37 °C for 10 min to visualize single-stranded or tetraplex DNA, respectively. Following incubation at 37 °C for 15 min and termination of the reaction, displaced single strands were resolved from their respective tetrahelical DNA structures by electrophoresis through a nondenaturing 12% polyacrylamide gel in 0.5 × TBE buffer, 20 mM KCl.](image)
tion of guanine quartets by WRN differs from that of unwinding of double-stranded DNA.

**Specificity and Efficacy of Tetraplex d(CGCG)n Unwinding by WRN**—Two helicases, BLM (28), and SV40 T-antigen (36) have been shown to unwind tetraplex DNA structures. However, the substrate specificity and efficiency of tetraplex unwinding by WRN differs from the unwinding of tetraplex DNA by these helicases. Both BLM (28) and SV40 T-antigen (36) were reported to unwind tetraplex structures of an IgG switch region sequence. By contrast, WRN failed to unwind tetraplex forms of this sequence (Fig. 5). In addition, whereas BLM was reported to unwind a tetrahelical structure of *Oxytricha* d(TTGGG)n telomeric sequence (28), WRN was unable to measurably unwind the G′2 tetraplex structure of the vertebrate telomeric sequence d(TTAGGG)n (Fig. 5). However, in unwinding a G′2 tetraplex 3′-tail d(CGCG)n, WRN acted more efficiently than either BLM or the SV40 helicase. Unwinding of G4 DNA by BLM and SV40 T-antigen was reported to reach completion within 20–45 min at enzyme to DNA ratios of 2:1 and 360:1, respectively (28, 36). WRN, however, fully unwound within 15 min G′2 3′-tail d(CGCG)n at a molar ratio of enzyme to DNA of 0.1 (Fig. 4).

**Possible Biological Significance of Unwinding of Tetraplex d(CGCG)n by WRN**—The recently reported capacity of BLM to unwind G4 structures of the immunoglobulin switch region and of *Oxytricha* telomeric sequence were interpreted as indicating a role of this helicase in the resolution of tetraplexes generated by strand invasion during DNA recombination or replication (28). The failure of WRN to unwind tetraplex structures of the IgG switch region or telomeric sequences suggests that it cannot replace BLM in resolving tetraplexes of specific sequence or structure. However, WRN efficiently unwound bimolecular quadruplex structures of d(CGCG)n. A d(CGCG) trinucleotide repeat was first identified in the 5′-untranslated region of the *FMR1* gene (37–39). The propensity of d(CGCG)n tracts to fold into hairpin structures and to assemble into tetraplex structures was implicated in the expansion of this sequence and the obstructed transcription and replication of *FMR1* in fragile X syndrome (25, 32, 33). Moreover, several expressed sequences from human genomic library were found to bear d(CGCG)n repeats (41). Notably, trinucleotide repeats other than d(CGCG) also readily fold into secondary structures (31). It is possible that hairpin or tetraplex structures of trinucleotide repeats form subsequent to their exposure as single-stranded stretches during DNA replication, transcription, or recombination. A potential function of WRN might be to unwind such secondary structures, thus relieving replication, transcription or recombination constraints. The slowed replication in Werner syndrome cells and accumulation of large deletions in their DNA might therefore be a reflection of defective removal of DNA secondary structures resulting from lack of WRN activity. The different DNA substrate specificities of the two human RecQ homologues, BLM and WRN, could be relevant to the distinctly different phenotypes of the two syndromes.

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