Residues in the RecQ C-terminal Domain of the Human Werner Syndrome Helicase Are Involved in Unwinding G-quadruplex DNA

The structural and biophysical properties typically associated with G-quadruplex (G4) structures render them a significant block for DNA replication, which must be overcome for cell division to occur. The Werner syndrome protein (WRN) is a RecQ family helicase that has been implicated in the efficient processing of G4 DNA structures. The aim of this study was to identify the residues of WRN involved in the binding and ATPase-driven unwinding of G4 DNA. Using a c-Myc G4 DNA model sequence and recombinant WRN, we have determined that the RecQ-C-terminal (RQC) domain of WRN imparts a 2-fold preference for binding to G4 DNA relative to non-G4 DNA substrates. NMR studies identified residues involved specifically in interactions with G4 DNA. Three of the amino acids in the WRN RQC domain that exhibit the largest G4-specific changes in NMR signal were then mutated alone or in combination. Mutating individual residues implicated in G4 binding had a modest effect on WRN binding to DNA, decreasing the preference for G4 substrates by ~25%. Mutating two G4-interacting residues (T1024G and T1086G) abrogated preferential binding of WRN to G4 DNA. Very modest decreases in G4 DNA-stimulated ATPase activity were observed for the mutant enzymes. Most strikingly, G4 unwinding by WRN was inhibited ~50% for all three point mutants and >90% for the WRN double mutant (T1024G/T1086G) relative to normal B-form dsDNA substrates. Our work has helped to identify residues in the WRN RQC domain that are involved specifically in the interaction with G4 DNA.

Helicases use the energy from ATP hydrolysis to catalyze the manipulation of nucleic acid substrates (1, 2). As such, they serve a variety of functions within the cell. Specialization of helicase function is a necessity arising from both the complexity of nucleic acid metabolism and the dynamic nature of DNA and RNA (3). Thus, it is not surprising that multiple types of helicases have been identified. Helicases have been divided into six superfamilies (SF1–6) based primarily on sequence homology (4). The RecQ enzymes are SF2 helicases that are conserved from prokaryotes through eukaryotes, with five human genes known to encode RecQ family members (5). Mutations in three of the five RecQ genes result in the following disease syndromes: Werner, Bloom, Rothmund-Thomson, RAPADILINO, and Baller-Gerold. The Werner syndrome protein (WRN),2 like all other RecQ family members, is an ssDNA-stimulated ATPase with 3′- to 5′-helicase activity (6, 7). Of the human RecQ family members, only WRN and the Bloom’s syndrome protein (BLM) have all three conserved RecQ domains as follows: 1) a core helicase domain composed of two RecA-like domains (HD1 and HD2); 2) the RecQ C-terminal (RQC) domain; and 3) the helicase and RNase D-like C-terminal (HRDC) domain. WRN is unique among the RecQ enzymes because it also possesses an N-terminal 3′- to 5′-exonuclease domain. Additional motifs and regions important for protein–protein interactions, as well as oligomerization states, have been reviewed thoroughly by others (5).

The cellular functions of RecQ helicases are diverse and include genomic replication, telomere maintenance, DNA repair, recombination, translesion synthesis, S-phase checkpoint activation, and involvement in the Fanconi anemia pathway. One feature that stands out regarding the cellular roles of WRN and BLM seems to be the repeated association of these enzymes with motifs capable of forming G-quadruplex (G4) structures. For example, both WRN and BLM were found to coordinate with the FANCJ helicase to increase the efficiency of replication across G4 motifs in DT40 avian cells (8). Cells derived from Werner syndrome (WS) patients exhibit a mutant phenotype, undergo premature replicative senescence, delayed S-phase progression, sensitivity to topoisomerase inhibitors and other genotoxic agents, as well as defects in telomere maintenance (9–13). Loss of WRN results in an

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2 The abbreviations used are: WRN, Werner helicase; BQQ, black hole quencher; BLM, Bloom helicase; FAM, 6-carboxyfluorescein; G4 DNA, G-quadruplex DNA; HRDC, helicase and RNase D-like C-terminal domain; HSQC, heteronuclear single quantum coherence; RH AU, RNA helicase associated with AU-rich element; RQC, RecQ C-terminal; TAMRA, carboxytetramethylrhodamine; ssDNA, single-stranded DNA; IPTG, isopropyl β-D-thiogalactopyranoside; β-ME, β-mercaptoethanol; WS, Werner syndrome.

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4 A summary of our previous work on the c-Myc G4 model sequence is provided in Supplemental Figs. S1 and S2.
increased mutation frequency for human telomeric sequences capable of forming G4 structures, as evidenced by results from supF mutagenesis assays (14). The increased mutation frequency in WRN-deficient cells was primarily attributable to an approximate 9-fold increase in the number of large deletions and rearrangements for the telomeric motif relative to the non-telomeric control sequence. In addition to replication defects, WS cells exhibit marked changes in the expression of genes that have G4 motifs near transcription start sites and at the 5’ end of the first intron (15–17). Biochemical experiments have shown that both WRN and BLM can bind to and unwind various G4-containing substrates (6, 18–20).

A number of helicases have been implicated in the unwinding and maintenance of G-quadruplex structures (21). However, the molecular features that impart certain helicases with the ability to effectively unwind G4 structures remain largely unknown. Previous studies have implicated the RQC domain in G4-specific activities of RecQ helicases (22), but the identification of amino acids that influence these interactions has remained elusive. We have investigated the role of the WRN RQC domain in binding and unwinding G4 DNA using biophysical and biochemical techniques. Our results provide insight into how specific amino acids in the RQC domain of WRN assist in the manipulation of G-quadruplex DNA substrates.

**Results**

Residues That Confer Preferential Binding to G4 DNA Reside in the RQC Domain of WRN—The RQC domain of WRN has previously been implicated in binding to DNA, a fact that was further supported by a co-crystal structure of the WRN RQC in complex with dsDNA (23, 24). The RQC domain is also critical for effective DNA unwinding activity, with three hydrophobic residues in the Tyr-1034, Phe-1037, and Met-1038) serving as a “separating knife” for the helicase (23). An earlier study had also implicated the RQC domain of BLM in binding to G4 DNA with high affinity (22). Because the aim of this study was to identify residues in WRN that allowed it to bind more favorably to G4 DNA over non-structured DNA, we wanted to confirm that this specificity also resided in the WRN RQC domain. To this end, different truncated WRN constructs were prepared (Fig. 1A), and the DNA binding properties of each were tested on both non-G4 and G4 DNA substrates (Fig. 1B and Table 1). The recombinant WRN proteins that contained the RQC domain (WRN(500–1092) and WRN(949–1092)) were found to bind G4 DNA with ~2-fold greater affinity than non-G4 DNA (Table 2). The core ATPase domain of WRN (amino acids 500–949; WRN(500–949)) exhibited lower overall affinity for the DNA substrates tested and lost the preference for G4 DNA, suggesting that the RQC domain was required to confer binding specificity toward G4 DNA to WRN.

**NMR Spectroscopic Studies Identify Residues in the WRN RQC Domain That Are Involved in G4-specific Interactions—**Because DNA binding results were consistent with a role for the RQC domain in conferring preference toward the G4 DNA substrate, we next sought to identify the individual residues of the WRN RQC domain that were involved in binding to G-quadruplex structures. Toward this goal, we expressed and purified...
TABLE 2
DNA binding affinities for truncated WRN constructs

| Protein            | $K_d$ DNA | $K_d$ Non-G4 DNA | $K_d$ DNA G4 | Preference for G4 DNA
|--------------------|-----------|-----------------|--------------|-------------------|
| WRN(500–1092)     | 514 ± 30  | 273 ± 5         | 1.9-Fold     |                     |
| WRN(949–1092)     | 440 ± 11  | 210 ± 4         | 2.1-Fold     |                     |
| WRN-(500–949)     | 890 ± 28  | 894 ± 8         | No preference|                   |

$^a$ Preference for G4 DNA substrates was calculated as ($K_d$ Non-G4 DNA/$K_d$ G4 DNA) - 1. Values represent the mean ± S.D. ($n = 3$).

$^{15}$N-labeled WRN RQC (WRN(949–1092)) and performed NMR spectroscopic studies on the protein, titrating in either non-G4 or G4 DNA substrates to observe changes in signal specific to each substrate (Fig. 2A and Table 1). The DNA substrate was added in increasing molar equivalents to the $^{15}$N-labeled RQC protein, and changes in peak intensity of the amide protons of individual residues were monitored in each spectrum. Many residues exhibited a decrease in peak intensity as a function of increasing DNA. The change in peak intensity was normalized and re-plotted as a function of DNA concentration (Fig. 2B), and the results were fit to a quadratic equation to obtain an estimate of the equilibrium dissociation constant ($K_d$). Although a quadratic equation is most appropriate for binding analyses, using a quadratic equation to fit changes in the NMR titration signal resulted in values that were difficult to interpret for some residues even when there were clear differences between G4 and non-G4 substrates. In some instances, we fit the data to a hyperbolic equation as a way of obtaining a semi-quantitative measure of RQC binding affinity for each DNA substrate. By comparing the affinity estimates for non-G4 and G4 DNA substrates, we were able to identify several residues in the WRN RQC that exhibit a >2-fold increase in affinity for G4 DNA relative to non-G4 DNA (Fig. 2C), indicative of a stronger interaction with the G-quadruplex-containing substrate. The 10 residues with the largest difference between the affinity for G4 and non-G4 DNA substrates were Phe-964, Leu-966, Arg-987, Phe-1004, Ala-1017, Ser-1019, Thr-1024, Thr-1044, Leu-1063, and Thr-1086 (Fig. 2D, yellow patches on cyan protein). We selected three residues (Thr-1024, Leu-1063, and Thr-1086) that were judged unlikely to disrupt the overall fold of the protein for site-directed mutagenesis. The mutant WRN enzymes were prepared using the construct possessing amino acid residues 500–1092 to examine the effect mutating these sites had upon G4-stimulated ATPase activity, G4 DNA binding, and unwinding of G-quadruplex structures.

Mutating More Than One G4-interacting Residue in the RQC Abrogates the Preference for WRN Binding to G4 DNA—We selected the following three amino acids in the WRN RQC as candidates for imparting G4-specific activities: Thr-1024, Thr-1086, and Leu-1063. DNA binding experiments were used as a first test of the effect of mutating these sites on the G4-specific activities of WRN. A modest decrease in the preferential binding to G4 DNA was observed for all three mutants (Table 3). Wild-type WRN(500–1092) bound G4 DNA 2-fold tighter than non-G4 DNA, where as each of the mutants (T1024G, L1063G, and T1086G) were found to bind G4 DNA 1.4–1.5-fold tighter than non-G4 DNA. Mutating two G4-interacting residues in the WRN RQC abrogated the preference for binding to G4 DNA. The double mutant (T1024G/T1086G) bound G4 and non-G4 DNA substrates with similar affinity, consistent with the notion that a cumulative effect imparted by multiple residues in the WRN RQC domain facilitate G4-specific binding.

Mutations in the RQC Domain Have Modest Effects on the G4 DNA-stimulated ATPase Activity of WRN—Next, we wanted to determine whether mutations in the WRN RQC domain affected the ATPase activity of the enzyme, and furthermore, whether there were G4-specific differences between the ATPase activity of each mutant protein as compared with WT. The ATPase activity of wild-type and mutant WRN was measured using two different DNA substrates: a non-G4 DNA control and a G4 DNA substrate. Both substrates were 42 nucleotides in length. The rate of ATP hydrolysis for wild-type WRN(500–1092) enzyme was similar on both G4 and non-G4 DNA substrates (Fig. 3A and Table 4). Mutating Thr-1086 reduced the ATPase activity of the enzyme, but there did not appear to be a G4-specific effect, as the mutant exhibited ATPase activity that was 69 and 62% that of wild-type enzyme on non-G4 and G4 DNA, respectively (Table 4). Likewise, the Thr-1086 mutation did not affect the ATPase activity of WRN on G4 DNA any more than it did that observed for the non-G4 DNA substrate (Table 4). The reduction in activity of the double mutant was ~30% greater for the G4 DNA substrate than that measured for non-G4 DNA (Fig. 3B and Table 4). Overall, the ATPase activity of the mutant enzymes did not appear to be affected in a G4-specific manner, although, similar to the DNA binding results, the double-mutant exhibited the largest G4-specific decrease.

Mutant WRN Proteins Showed Significantly Compromised Unwinding Ability Compared with Wild-type Enzyme on c-Myc-derived G4 DNA Substrates—We measured the DNA unwinding activity of each mutant protein on both non-G4 and G4 DNA substrates. Helicase activity was monitored using an assay that relies on DNA substrate possessing a TAMRA-labeled “loading” strand (i.e. the strand with an ssDNA overhanging or loading zone for the helicase) and a BHQ2-labeled displaced strand. The displaced strand was designed to form a 10-bp region of dsDNA near the 5’ end of the loading strand (Table 1). The G4 motif was positioned directly adjacent to the dsDNA region. Unwinding of the G4 structure must occur before the BHQ2-labeled strand can be displaced. A substrate with a non-G4 DNA loading strand was prepared as a control. The increase in TAMRA fluorescence over time was measured, and the initial portion of the velocity curve was used to calculate an unwinding rate (Fig. 4A). The unwinding activities of the mutant enzymes were compared with that of wild-type WRN(500–1092) to assess G4-specific effects.

Percent activity was determined by normalizing the unwinding rate for each mutant protein to the unwinding rate measured for wild-type WRN(500–1092) (defined as 100%). Separate experiments were performed using non-G4 and G4 DNA-containing substrates. All four mutants exhibited unwinding activity comparable with wild-type WRN on non-G4 DNA substrates (Fig. 4, B and D). However, with the G4 DNA, results were dramatically different. All mutant proteins showed a reduced efficiency of unwinding G4 DNA when compared to WT, with the...
single mutant WRN<sup>T1086G</sup> exhibiting the smallest effect on G4 activity (~40% reduction relative to WT) and WRN<sup>T1024G</sup> exhibiting the largest decrease in G4 activity of the single mutants (~60% reduction relative to WT). However, the most dramatic reduction in G4 DNA unwinding ability was seen with the double mutant WRN<sup>T1024G/T1086G</sup>, which exhibited a >90% reduction in helicase activity on the G4 substrate. These results demonstrated the important role that these residues

**FIGURE 2. Identification of G4-specific amino acids in the WRN RQC domain.** A, NMR spectra resulting from HSQC experiments titrating G4 DNA into a solution of <sup>15</sup>N-labeled WRN RQC (amino acids 949–1092) domain are shown. Changes in peak intensity were monitored and quantified. B, peak intensity for each residue was monitored as a function of increasing amounts of DNA added and fit to a quadratic equation to estimate the apparent binding affinity ($K_d$) for each residue. C, $K_d$ values for non-G4 (red bars) and G4 (blue bars) DNA substrates for Thr-1024 and Leu-1063 are shown. D, mean structure of the WRN RQC derived from NMR experiments (Protein Data Bank code 2AXL) was superimposed upon coordinates derived from the crystal structure of WRN RQC bound to dsDNA (Protein Data Bank code 3AAF). The 10 amino acids with the largest $K_{app, G4 DNA}/K_{app, non-G4 DNA}$ (i.e., greatest affinity for G4 DNA) are shown (yellow). The α-helices, the α2-α3 loop, the strand-separating β-wing, and the unstructured C-terminal region of the WRN RQC domain are also labeled.
A  wild-type WRN

B  WRN

FIGURE 3. ATPase activity of WRN RQC mutant enzymes on G4 DNA is not greatly perturbed. The ATPase activity of wild-type (A) and T1024G/T1086G WRN(500–1092) (B) was measured using a coupled spectrophotometric assay (see “Experimental Procedures”). Results are shown for both G4 (blue squares) and non-G4 (red circles) DNA substrates.

Discussion

RecQ helicases have been implicated in unwinding G-quadruplex structures based on biochemical/biophysical studies, as well as compelling biological results. Recent reports have demonstrated the distinct roles of the HRDC and RQC domains of BLM in interacting with intra-strand G4 DNA structures (25). To date, however, identifying the molecular determinants important for WRN interactions with G4 DNA sequences has remained an elusive goal. Our study aimed to address this shortcoming by specifically focusing on residues that are involved in WRN-catalyzed unwinding of G4 DNA.

We were able to confirm that the G4 DNA-specific binding by WRN is dependent on the presence of the RQC domain (Fig. 1 and Table 2). It is quite possible, as seen in the case of BLM (mentioned above), that other domains like the HRDC or exonuclease domain play additional roles in the G4-related properties of WRN. Our results show that the RQC domain alone is able to confer an ∼2-fold binding preference to G4 DNA over non-G4 DNA sequences. These results supported conclusions drawn from other studies implicating the RQC domain in G4-related activities of RecQ helicases, but they did not identify amino acid residues involved in the G4 specific activities of WRN.

Further insights into the molecular determinants of WRN RQC interactions with G4 DNA were obtained from our NMR spectroscopy results (Fig. 2). Residues Thr-1024, Leu-1063, and Thr-1086 exhibited the largest perturbations in their chemical environment upon addition of G4 DNA substrate relative to non-G4 DNA. Interestingly, none of the strand-separating...
FIGURE 5. **WRN RQC double mutant fails to unwind telomeric G4 DNA.** A, schematic illustration of the FRET-based unwinding assay for the telomeric G4 DNA substrate (Tel22) is shown. B, change in Cy3 fluorescence over time was monitored for both wild-type (black circles) and T1024G/T1086G (red squares) WRN(500–1092). C, rate of Tel22 unwinding by wild-type (black) and T1024G/T1086G WRN(500–1092) was compared with that observed for the non-G4 DNA substrate to estimate the relative helicase activity of each enzyme against telomeric G4 DNA. The results shown represent the mean ± S.D. (n = 3). The reported p value was calculated using an unpaired Student’s t test comparing wild-type and mutant WRN.

FIGURE 4. **Point mutations in the WRN RQC domain impair unwinding of c-MYC G4 DNA.** A, schematic illustration of the helicase assay used to monitor unwinding of the c-MYC G4 DNA structure is shown. A control substrate containing a non-G4 sequence was used for comparison. B, helicase activity for wild-type (bright red) and T1024G/T1086G (dark red) WRN(500–1092) is shown for the non-G4 DNA substrate. C, helicase activity for wild-type (bright blue) and T1024G/T1086G (dark blue) WRN(500–1092) is shown for the c-MYC G4 DNA substrate. D, relative unwinding rate (mutant rate/wild-type rate × 100) on the non-G4 DNA substrate is plotted for each of the WRN RQC mutants. E, relative unwinding rate (mutant rate/wild-type rate × 100) on the c-MYC G4 DNA substrate is plotted for each of the WRN RQC mutants. The results shown represent the mean ± S.D. (n = 3). All p values were calculated using an unpaired Student’s t test comparing wild-type and mutant WRN.
hydrophobic residues in the β-wing of WRN (Tyr-1034, Phe-1037, and Met-1038) were identified as G4-specific interactors. It is possible that the crucial role of these residues in the helicase activity of WRN on canonical dsDNA substrates precludes any G4-specific interactions. Additionally, our NMR titration also failed to identify residues in the α2-α3 loop as G4-specific binders. Again, this is presumably due to the fact that residues in the α2-α3 loop recognize and bind to the phosphates in B-form dsDNA (23).

The three residues examined in our study, Thr-1024, Leu-1063, and Thr-1086, are located in the α4 recognition helix and the C-terminal unstructured region of the WRN RQC (Fig. 2E). The Leu-1063 position is part of the β-sheet in the WRN RQC crystal structure, but this region of the protein adopts a less structured element in the NMR study. The crystal structure of the WRN RQC bound to dsDNA positions the C-terminal end of the α4-recognition helix (including Thr-1024) ~15 Å away from the dsDNA substrate (23). The C terminus and β-sheet are not crucial for the integrity of the core RQC domain and are not in close proximity to the DNA substrate when the NMR structure, which includes the disordered C terminus containing both Leu-1063 and Thr-1086, was superimposed onto the crystal structure (26). The integrity of the truncated RQC domain suggests that some structural flexibility in the C-terminal amino acids is tolerated, which could be important for contacting G4 DNA structures that can adopt multiple folding intermediates. In a general sense, the residues identified in this study form a G4-binding surface that is largely distinct from the residues in the WRN RQC observed previously to bind and unwind the blunt-end dsDNA substrate.

Functionally, mutating the three amino acids noted above led to modest decreases in ATPase activity on both non-G4 and G4 DNA substrates. The decrease in ATPase activity on G4 DNA was most pronounced for the double mutant WRN(T1024G/T1086G), where the decrease relative to WT was ~30% greater for the G4 substrate than that observed for the non-G4 DNA substrate (Fig. 3 and Table 4). This suggests that the ability to hydrolyze ATP per se may not be compromised in this mutant, but its ability to interact with G4 DNA might be inefficient, as compared with WT. This was borne out in the DNA binding experiments with the mutants, as the double mutant exhibited the greatest drop in specificity toward binding to G4 DNA as compared with WT (Table 3). Thus, these residues seem to play a role imparting binding preference toward G4 DNA to WRN.

The most dramatic effects of mutating Thr-1024, Leu-1063, or Thr-1086 were observed when we tested the ability of these WRN mutants to unwind the c-MYC G4 DNA structure. Compared with the WT protein, G4 DNA unwinding efficiency dropped progressively from WRN(T1086G) (40%) to WRN(T1086G) (50%) to WRN(T1024G) (60%) on G4 DNA substrates derived from the c-MYC promoter (Fig. 4). The largest effect was seen with the double mutant (drop in unwinding activity by ~90%). None of the mutant proteins were significantly affected in their ability to unwind non-G4 DNA, as compared with WT.

The parallel-stranded c-MYC G4 DNA structure represents one of the most stable G-quadruplexes studied to date, with a melting temperature of 74 °C (27–29). We tested the ability of the mutant WRN proteins to unwind a less stable G4 structure in the form of the telomeric sequence 5′-T-TGGG- (TTAGGG)_nT-3′ (30). Similar to the c-MYC G4 substrate, mutating Thr-1024 and Thr-1086 to Gly abrogates ~90% of G4 unwinding relative to the wild-type enzyme (Fig. 5).

Recently, a single-molecule imaging study investigated differences in the G4 unwinding mechanisms of the following three RecQ enzymes: BLM, WRN, and RNA helicase AU (associated with AU-rich elements) (RHAU, also called DHX36 or G4 resolvase 1) (31). ATP-dependent repetitive unfolding of telomeric G4 DNA (but not the c-MYC G4 substrate) was observed for WRN. The authors interpreted these results to mean that WRN does not interact with or unwind the parallel-stranded c-MYC G4 structure. One caveat to the interpretation of the results presented in the single molecule imaging study is related to the fact that WRN binding to the c-MYC G4 substrate relied upon changes in FRET that presumably accompany ATP-driven unfolding of the G4 structure by WRN. Because these experiments were performed in K+ (25 mM), it seems very likely that the c-MYC G4 structure was too stable for WRN helicase action to overcome. Indeed, we do not observe WRN-catalyzed unwinding of the K+ stabilized c-MYC 2345 G14/23T substrate (data not shown). By preparing the c-MYC G4 substrate in Na+, we were able to monitor WRN-catalyzed unwinding (Fig. 4). By way of comparison, the telomeric G4 DNA substrate used in our assays was unwound in an ATP-dependent manner by WRN even when prepared in K+ (Fig. 5). Therefore, it is probably safe to assume that WRN is less capable of unwinding the more stable G4 structure adopted by c-MYC sequences when compared with the activity observed for BLM and RHAU. In contrast, the telomeric G4 sequences appear to be readily unwound by WRN, consistent with the defects in telomere maintenance observed for WS cells.

The relative importance of WRN-mediated G4 activity in the manifestation of WS is difficult to assess, especially given the wide-ranging role of the enzyme in multiple aspects of nucleic acid metabolism. There are certainly deletion mutations in WS patients that eliminate the G4-interacting residues examined here (e.g. 3265–3266delGA and 3259–3262delCAA) (32). However, there are only a handful of WS patients identified with missense mutations, and none of those patients have missense variants for residues Thr-1024, Leu-1063, or Thr-1086. The Thr-1024 residue, although not conserved itself, resides between a set of hydrophobic amino acids in the α4-helix (i.e. Leu-1022, Phe-1027, and Leu-1028) that are conserved from the bacterial RecQ helicase to eukaryotic RecQ enzymes. Yeast Sgs1 and *Xenopus laevis* WRN retain a threonine corresponding to human Thr-1024, but otherwise, this residue is not conserved across species. The human WRN Leu-1063 residue is invariant between mouse, chicken, and *Xenopus* WRN. It also appears to be conserved in bacterial RecQ (Leu-510) and highly conserved among other RecQ proteins. It remains to be seen whether the G4 unwinding capacity of other RecQ enzymes is affected by mutating residues corresponding to human WRN Thr-1024 or Leu-1063.

Although WRN and other RecQ enzymes have demonstrated roles in G4 biology, there are clearly additional helicases of great importance when it comes to G-quadruplex mainte-
nance. Foremost among these enzymes is the FANCJ helicase, which was recently shown to have tight binding affinity and robust unwinding activity with G4 substrates (33). In contrast to the preferential G4 binding observed with WRN, FANCJ bound both G4 and non-G4 substrates with a $K_d$ near 1 nM (33). Similar to WRN and BLM, FANCJ-deficient cells exhibit large deletions near sites in the genome that are rich in G4 motifs (34). As noted before, FANCJ and the RecQ members BLM and WRN appear to function in parallel to unwind G4 structures during replication (8). Alternatively, FANCJ may act in concert with the translesion polymerase Rev1 to facilitate G4 resolution. This is interesting for a variety of reasons, including the fact that FANCJ exhibits 5′- to 3′-directionality on DNA, which would place it in an opposing orientation to replication fork progression (i.e. Rev1 action) and RecQ helicases, which translocate with 3′- to 5′-directionality. The structural basis for FANCJ activity on G4 substrates is unknown. The AKKQ motif in the FANCJ iron-sulfur domain appears to be important for G4 recognition and unwinding. Mutating the lysine residues in this motif (Lys-141 and Lys-142) produce defects in the G4 unwinding activity of FANCJ (33). The RecQ enzymes do not appear to possess the AKKQ motif. However, superimposition of the G4 recognition peptide from the RHAU (DHX36) helicase reveals a striking similarity with the α4-helix of the WRN RQC (supplemental Fig. S2). The 18-amino acid G4 recognition α-helix from RHAU adopts a L-shaped configuration (35). This configuration is mirrored by a strong kink near the N-terminal portion of the α4-helix in the WRN RQC (supplemental Fig. S2). Importantly, many of the residues that exhibited the most pronounced G4-specific interactions are clustered near this site on the WRN RQC (supplemental Fig. S2). This “corner” of the WRN RQC extends out above and slightly beyond the α2-helix (Fig. 2). If we compare the RQC to the G4 interface observed for the RHAU peptide, the presence of the α2-helix would seem to limit the G4-interacting region of the L-shaped α4-helix. Such a scenario would fit with a role for additional G4-specific residues we identified in the flexible C-terminal portion of the WRN RQC, which may serve as additional points of contact between WRN and the G4 substrate.

In conclusion, this study has shed light on the role played by specific residues of WRN involved in the binding and resolution of G4 DNA sequences. The residues are located in a region of the RQC domain that is adjacent to the strand-separating β-wing. Moreover, G4-interacting residues in the flexible C-terminal portion could adopt multiple structures to accommodate the dynamic nature of G-quadruplexes, which could facilitate unwinding by allowing WRN to maintain contact with the G4 sequence during the multistep process of tetrads disruption.

### Experimental Procedures

**Materials**—Unlabeled deoxyribonucleoside triphosphates (dNTPs) were obtained from GE Healthcare. Oligonucleotides used in this work, with the exception of the BHQ2- and TAMRA-labeled oligonucleotides, were synthesized by Integrated DNA Technologies (Coralville, IA) and purified using high performance liquid chromatography (HPLC) by the manufacturer, with analysis by matrix-assisted laser desorption-time of flight mass spectrometry. The 47-mer 5′-BHQ2-labeled oligonucleotides and the 10-mer 3′-TAMRA-labeled oligonucleotide used in the helicase assay were synthesized by Biosearch Technologies (Novato, CA). G4 DNA substrates were prepared as described previously (36).

**Protein Expression and Purification**—The expression plasmids containing the coding regions for WRN(500−1092) and WRN(949−1092) as N-terminal His$_6$-GST fusion constructs were obtained from DNA 2.0 (Menlo Park, CA). The truncated constructs WRN(500−1092), WRN(500−949), and WRN(949−1092) (RQC) were generated by introducing a stop codon by site-directed mutagenesis. The mutant proteins for WRN(500−1092) with single point mutations T1024G, L1063G, T1086G, and the double mutant T1024G/T1086G were generated through site-directed mutagenesis of the WRN(500−1092) plasmid. Mutant plasmids were confirmed by DNA sequencing.

All WRN proteins were overexpressed in *Escherichia coli* BL21 (DE3) Gold cells (Agilent Technologies, Santa Clara, CA). Bacterial cultures were grown in LB medium containing 50 μg/ml kanamycin by incubating in a shaker at 37 °C for 3 h ($A_{600} = 0.6 – 0.8$). Next, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.7 mM for induction, and the cultures were grown at 22 °C overnight. Cells were harvested the next day; pellets were washed and resuspended in 50 mM Tris-Cl buffer (pH 7.4) containing 300 mM NaCl, 5 mM β-mercaptoethanol (β-ME), and 10% glycerol containing protease inhibitor mixture (Pierce) and 1 mg/ml lysozyme (Buffer A). Cells were lysed by sonication, and clarified lysate was recovered after ultracentrifugation (35,000 × g, 1 h, 4 °C). The protein was purified from filtered lysate by affinity chromatography, by loading the clarified lysate onto a nickel-Sepharose column. Contaminating proteins were removed by sequential washes with Buffer A containing 20 and 75 mM imidazole. The bound WRN protein was eluted using a buffer containing 400 mM imidazole. Peak fractions containing purified protein were pooled and concentrated, and the imidazole-containing buffer was exchanged using Amicon centrifugal concentrators (EMD-Millipore). The purified protein was then loaded onto a glutathione-Sepharose affinity column in 25 mM HEPES buffer (pH 7.5) containing 200 mM NaCl, 5 mM β-ME, and 10% glycerol (Buffer B). The N-terminal His$_6$-GST tag was cleaved by overnight incubation in buffer containing HRV3C protease (Pierce) while bound on the column, followed by elution of the affinity tag-free WRN protein in Buffer B. This was further concentrated and subjected to a polishing step on a Superdex 200 size exclusion column. Protein purity was estimated using SDS-PAGE and circular dichroism (CD) (supplemental Fig. S1). CD measurements were carried out on a Jasco J-715 spectropolarimeter at 25 °C. Far-UV spectra (between 190 and 240 nm) were collected with scan speed of 20 nm/min, at a bandwidth of 1 nm and a response time of 4 s. Each spectrum was the average of five scans, and for each protein, the buffer/blank spectrum was subtracted to get the spectrum for each protein alone. The WRN(500−1092) WT and four mutant proteins were used at 0.3 mg/ml (4.5 μM) in 25 mM phosphate buffer, pH 7.4, containing 20 mM NaCl. A quartz cell of a path length of 0.1 cm was used for collecting all CD spectra, and the acquired data were expressed as mean molar residue ellipticity in degrees cm$^2$ dmol$^{-1}$. Fractions of
highly pure WRN proteins were concentrated, and glycerol was increased to 25% (v/v) and was stored at −80 °C.

Expression and Purification of 15N-Labeled RQC Protein for NMR Studies—Small scale (5 ml) cultures of the RQC construct were grown in LB medium by inoculating colonies of RQC-transformed BL21 (DE3) Gold cells for 12 h at 37 °C. The cells were pelleted and washed with sterile phosphate-buffered saline three times to remove traces of the medium. The pellets were then transferred to culture flasks containing sterile M9 minimal medium that was supplemented by 15N-ammonium chloride (Cambridge Isotopes Laboratories, MA) as the sole source of nitrogen. Cultures were grown in the M9 medium at 37 °C for 5 h (A600 = 0.6 – 0.7), following which IPTG was added to a final concentration of 0.7 mM, and the cultures were grown further at 30 °C for 5 h to allow induction of protein expression. Cells were then harvested by centrifugation at 5000 rpm (6238 × g) at 4 °C for 15 min; the pellet was washed in Buffer A, and the 15N-labeled RQC protein was purified from the lysate as described before. After obtaining >95% pure 15N-labeled RQC protein after the final polishing step (Superdex 200), the protein was concentrated and further transferred to 25 mM potassium phosphate buffer (pH 7.4) containing 25 mM KCl, 100 mM NaCl, 4 mM dithiothreitol, 1 mM EDTA, and 7.5% (v/v) deuterium oxide (NMR buffer). Final concentration of the protein was 0.25 mM.

NMR Titration Studies of 15N-Labeled WRN-RQC with Non-G4 and G4 DNA—NMR spectroscopic experiments were performed at 25 °C at 600, 800, and 900 MHz using Bruker AVIII NMR systems, each equipped with a CPTCI probe at the Vanderbilt Biomolecular NMR Facility. The 15N-labeled WRN-RQC protein was at a concentration of 0.25 mM in the NMR buffer containing 7.5% D2O. Increasing molar equivalents of non-G4 or G4 DNA (11-/-28-mer dsDNA) were titrated into the protein solution, and the resulting 1D proton and 2D 1H/15N HSQC NMR spectra were recorded. Individual peak assignments were made for 75 of the 143 WRN RQC amino acid residues based on the published data deposited in BMRB data bank, accession 6540. Changes in peak intensity for these residues were monitored as a function of increasing amounts of DNA added and were plotted and fit to a hyperbola to obtain a semi-quantitative estimate of the apparent binding affinity (Kapp) for each residue. The molar ratio of RQC/DNA ranged from 1:0.015 (i.e. a 67-fold molar excess protein) to 1:2.3 (i.e. a 2.3 M excess of DNA over protein). Integrated peak intensity was measured at 12 different RQC/DNA molar ratios. By performing identical experiments with both non-G4 and G4 DNA substrates, affinity estimates were obtained for both substrates by plotting the change in peak intensity as a function of DNA concentration and fitting the results to a quadratic equation in some instances, the resulting plot could not be fit to a quadratic equation, in which case a hyperbolic equation was used to obtain a semi-quantitative estimate of binding affinity. The relative affinity was then expressed as a ratio of non-G4/G4 DNA. This ratio was used to represent the degree to which a residue preferentially interacted with G4 DNA.

Spectrophotometric Measurement of ATPase Activity—Hydrolysis of ATP by all the ATPase domain-containing WRN proteins (WRN(500–949) and WRN(500–1092) and mutants thereof) was measured using a coupled spectrophotometric assay that measures the change in absorption by NADH as a function of molecules of ATP hydrolyzed using a pyruvate kinase–lactate dehydrogenase regeneration system. For ATPase assays, WRN (2–5 μM) was added to 25 mM HEPES buffer (pH 7.5) containing 10 mM potassium acetate, 10 mM magnesium acetate, 2 mM β-ME, 50 μM EDTA, 5 mM ATP, 4 mM phosphoenolpyruvate, 1 mM NADH, and 10 units/ml pyruvate kinase–lactate dehydrogenase. Reactions were initiated by addition of 10 μM ssDNA (42-mer non-G4 or G4). ATP hydrolysis rates were determined at 25 °C by measuring the conversion of NADH to NAD+ at 380 nm (ε380 of NADH is 1210 M−1 cm−1). The oxidation of 1 mol of NADH corresponds to the hydrolysis of 1 mol of ATP.

Determination of DNA Binding Affinity by Fluorescence Polarization—DNA binding affinity was estimated on the non-G4 and G4 versions of both ssDNA and dsDNA substrates. The 5'-FAM-labeled 28-mer non-G4 and G4 oligonucleotides were used for ssDNA binding studies, whereas the dsDNA substrates used in these assays were generated by annealing the FAM-labeled 28-mer oligonucleotide to the 11-mer unlabeled complementary oligonucleotide in a molar ratio of 1:2. Each DNA substrate (1.5 nm) was incubated with varying concentrations of WRN (0 – 4 μM) in a 50 mM HEPES buffer (pH 7.5) containing 10 mM KOAc, 10 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 2 mM β-ME, and 0.1 mg/ml bovine serum albumin. The resulting change in fluorescence polarization units was plotted as a function of protein concentration, and the resulting curves were fit to a quadratic equation to determine the equilibrium dissociation constant for binding of the WRN proteins to DNA (Kd, DNA).

Helicase Assays—The ability of the WRN(500–1092) WT and mutant proteins to unwind non-G4 and G4 DNA substrates was measured using a fluorescence-based helicase assay. DNA substrates for the helicase assay were prepared by annealing the 47-mer 5’-BHQ2-labeled template (non-G4 or G4) to the 10-mer 3’-TAMRA-labeled displaced strand in a molar ratio of 1:1.1 in 20 mM HEPES buffer (pH 7.5) containing 10 mM KCl. The Tel22 G4 DNA substrate was prepared in 20 mM HEPES buffer (pH 7.5) containing 100 mM KCl by heating the oligonucleotide to 95 °C and then slow cooling to room temperature. Reaction mixtures were prepared by mixing WRN (500 nm final concentration) in 20 mM HEPES buffer (pH 7.5) containing 20 mM NaCl, 2 mM KCl, 0.01% Tween 20, 4 mM β-ME, and 0.1 mg/ml bovine serum albumin with DNA substrate (75 nm) and aliquoted in the wells of a 96-well microfluor plate. Reactions with the Tel22 G4 DNA substrate were performed in the same buffer as those performed with the c-MYC-derived G4 substrate with the following modifications: 100 mM KCl (no NaCl) was included, and the DNA concentration was 50 nm. Reactions were initiated by adding Mg2+ (10 mM), ATP (100 μM), and ssDNA trap (375 nm) to each well. The sequence of the ssDNA trap used in reactions with the c-MYC substrate was 5’-GCA AAA AAA A-3’, and the sequence of the trap used in the reactions with Tel22 was 5’-CCC TAA CCC TAA CCC TAA CCC-3’. The increase in the fluorescence signal was monitored as a function of time, and the initial portion of the velocity curve was used to calculate the rate of helicase activity. All values were expressed as percent activity by normalizing to the
rate of unwinding obtained for the wild-type WRN protein. All unwinding experiments were performed in triplicate, and the results shown represent the mean ± S.D. (n = 3).

**Author Contributions**—R. L. E. designed the research. A. K., M. V., and T. M. performed the experiments. A. K. and R. L. E. analyzed the experimental results. R. L. E. wrote the paper. A. K., M. V., and T. M. provided comments on the manuscript. All authors approved the final version of the manuscript.

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