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The Enzymatic Activities of the Werner Syndrome Protein Are Disabled by the Amino Acid Polymorphism R834C*

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The Werner syndrome protein, WRN, is a member of the RecQ family of DNA helicases. It possesses both 3′→5′ DNA helicase and 3′→5′ DNA exonuclease activities. Mutations in *WRN* are causally associated with a rare, recessive disorder, Werner syndrome (WS), distinguished by premature aging and genomic instability; all are reported to result in loss of protein expression. In addition to WS-linked mutations, single nucleotide polymorphisms, with frequencies that exceed those of WS-associated mutations, are also present in *WRN*. We have initiated studies to determine if six of these polymorphisms affect the enzymatic activities of WRN. We show that two common polymorphisms, F1074L and C1367R, and two infrequent polymorphisms, Q724L and S1079L, exhibit little change in activity relative to wild-type WRN; the polymorphism, T172P, shows a small but consistent reduction of activity. However, an infrequent polymorphism, R834C, located in the helicase domain dramatically reduces WRN helicase and helicase-coupled exonuclease activity. The structure of the *E. coli* helicase core suggests that R834 may be involved in interactions with ATP. As predicted, substitution of Arg with Cys interferes with ATP hydrolysis that is absolutely required for unwinding DNA. R834C thus represents the first missense amino acid polymorphism in WRN that nearly abolishes enzymatic activity while leaving expression largely unaffected.

Helicases are enzymes that separate the complementary strands of nucleic acids. They are ubiquitous in nature and participate in numerous nucleic acid transactions, including replication, repair, and recombination. They are classified based on their substrate preference, the presence of signature helicase motifs, and the directionality of unwinding (1, 2).

The RecQ family of DNA helicases unwinds duplex DNA with 3′→5′ polarity (the directionality being defined by the DNA strand that is bound by the helicase). The energy required for unwinding is derived from hydrolysis of ATP. The prototype of this family is the *Escherichia coli* RecQ protein. Prokaryotes, including *E. coli*, as well as lower eukaryotes have a single RecQ family member, whereas higher eukaryotes have multiple members. For example, human cells have at least five RecQ helicases: RecQ1, BLM, WRN, RecQ4, and RecQ5. The function of each of these helicases in DNA metabolic processes is conjectural. However, mutations in three of the genes, *BLM*, *WRN*, and *RecQ4*, are associated with Bloom syndrome, Werner syndrome (WS),[1] and Rothmund-Thomson syndrome, respectively. All three disorders are characterized by genetic instability, implicating a central role of these DNA helicases in one or more DNA metabolic pathways (3, 4).

The WRN protein is 1432 amino acids long; its central domain has the RecQ consensus helicase motifs, I, Ia, and II–VI (5). *In vitro*, purified WRN, like *E. coli* RecQ, exhibits DNA-dependent ATPase activity and ATP-dependent 3′→5′ DNA unwinding activity (6). In addition, it exonucleolytically degrades DNA with 3′→5′ polarity, an activity unique to WRN among RecQ helicases (7).

Mutations in *WRN* are causally associated with WS, an uncommon recessive human disease characterized by premature aging and genomic instability (8). Clinical manifestations of WS, including atherosclerosis, osteoporosis, diabetes mellitus, and bilateral cataracts, and an increased incidence of non-epithelial cell-derived cancers are observed in early adulthood; death commonly occurs in the fourth decade of life (8, 9). Cultured cells from WS patients also exhibit reduced replicative lifespan (10) and increased genetic instability (11, 12). Genetic instability is manifested at the cytogenetic level by chromosome breaks and translocations, and at the molecular level by multiple, large DNA deletions (11, 12).

Disease-linked mutations are found throughout the *WRN* gene and include stop codons, splice site variants, and insertions/deletions that generate frameshift mutations (13–15). No missense mutations in *WRN* have been detected in WS patients. All identified *WRN* mutations predict the synthesis of truncated protein products lacking the C-terminal nuclear localization signal. Based on this observation, it has been proposed that the lack of a nuclear localization signal is important in the pathogenesis of WS (16). However, cell lines derived from individuals with WS have no detectable WRN (17), suggesting that WS may result from lack of WRN *per se*.

Sequencing efforts by the genome centers at the University of Washington (egp.gs.washington.edu) and Stanford University (18) have resulted in the identification of a large number of polymorphic amino acid substitutions in WRN. A total of 14 non-synonymous polymorphisms were reported at the time our studies were initiated. Since then, an additional six non-synonymous coding polymorphisms have been identified. Epidemi-

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[1] The abbreviations used are: WS, Werner syndrome; LCL, lymphocytic cell line; ATPγS, adenosine 5′-*O*-(thiotriphosphate).

ological studies have been carried out on the two most common polymorphisms, F1074L and C1367R. There are reports of an age-dependent decline of the 1074Phe/Phe genotype in both Finnish and Mexican populations, suggesting a beneficial effect of the Leu allele in aging populations (19). Also, the more frequent Cys allele at amino acid 1367 has been reported to be associated with a lower frequency of osteoporosis in post-menopausal Japanese women (20), whereas the rarer Arg allele may be associated with a lower risk of myocardial infarction in the Japanese population (21). No such correlative studies have been reported for any of the other WRN polymorphisms. Furthermore, neither biochemical studies nor functional analyses of any of the WRN variants have been reported.

To determine whether non-synonymous coding polymorphisms in *WRN* affect its biochemical properties, we expressed and characterized the activities encoded by six variant proteins. We found that one polymorphism that substitutes Arg at codon 834 with Cys dramatically reduces both WRN helicase and exonuclease activities without a major effect on WRN expression. A cell line heterozygous for R834C has ~50% of WRN helicase/exonuclease activity relative to the wild-type. Genotypic analyses of a large collection of anonymous DNA samples showed that this polymorphism is preferentially present in individuals of Spanish ancestry.

EXPERIMENTAL PROCEDURES

Growth of Cells

The kidney epithelial tumor cell line, 293T, was grown in Dulbecco's modified minimal essential medium (Cellgro) supplemented with 10% fetal bovine serum (HyClone), 2 mM L-glutamine, 100 units/ml penicillin G sulfate, and 100 μg/ml streptomycin sulfate (Invitrogen). Epstein-Barr virus-transformed lymphocytic cell lines (LCLs) from the Polymorphism Discovery Resource collection were obtained from the Coriell Cell Repository and were grown as suspension cultures in RPMI 1640 medium containing 15% fetal bovine serum and glutamine plus penicillin-streptomycin at the concentrations mentioned above. All cultures were grown in a humidified, 5% CO_2 incubator at 37 °C. 293T cells were used in transient transfection assays (see below). LCLs were used to assay helicase/exonuclease activity of endogenous WRN; cells were propagated by dilution into fresh medium and harvested at densities of 1–2 × 10^6 cells/ml.

WRN DNA Constructs

Nucleotide changes corresponding to each polymorphic site were introduced into the *WRN* coding sequence using mutagenic primers and *Pfu Turbo Taq* DNA polymerase as summarized in the QuikChange XL-site directed mutagenesis kit (Stratagene). Where feasible, silent nucleotide alterations were also introduced into the primers to create restriction sites that facilitated easy screening of mutagenized plasmids. Following PCR amplification, the DNA was digested with DpnI to remove methylated and hemimethylated DNA. DNA resistant to DpnI cleavage was electroporated into electrocompetent XL1 Blue cells. Plasmid DNA was isolated and screened by digestion with the engineered restriction enzymes and/or fragments spanning the mutated sites sequenced to verify the presence of the nucleotide change. These fragments were sub-cloned into a non-mutagenized vector. Plasmid DNA sequence was verified again following sub-cloning. The pCS2+MT vector (22) was used for sub-cloning and transient transfection. Expression is driven from the strong enhancer/promoter, simian CMV IE94, and results in the synthesis of WRN with six myc epitope tags at the N terminus.

The WRN cDNA clone used in the site-directed mutagenesis protocols contained the common polymorphism F1074L (frequency 0.41; egp.gs.washington.edu). Thus, all other polymorphisms were introduced in this background. The effects of these variants on WRN enzymatic activity were therefore compared with that conferred by F1074L alone. The effect of the leucine substitution was determined by replacing it with phenylalanine, the amino acid present in the remaining ~60% of the population; this construct is referred to as wild-type.

Transfection of WRN Constructs

DNA encoding each of the six alleles was prepared using the Qiagen Maxi Prep DNA purification kit. The DNA was ethanol-precipitated twice; A_{260}/A_{280} ratios were routinely 1.8. 293T cells were transfected with plasmid DNA encoding either wild-type or variant WRN. Expression of N-terminal myc epitope-tagged WRN from the plasmid is driven from the strong cytomegalovirus promoter. Cells were seeded at a density of 1×10^6 per 60-mm dish. 24 h later, 3 μg of plasmid DNA was introduced into cells by precipitation of DNA with calcium phosphate in HEPES. The medium was replaced after 12 h, and cells were harvested 36 h post-transfection. The cells were rinsed with phosphate-buffered saline, quick frozen in liquid nitrogen, and stored at −80 °C until use.

Preparation of Cell Lysates and Immunoprecipitation of WRN

Transfected 293T cells and LCLs expressing various WRN alleles were lysed for immunoprecipitation as described previously (23). WRN in lysates (500 μg of total protein) of transfected 293T cells was immunoprecipitated with a myc epitope-specific monoclonal antibody (9e10 monoclonal antibody; kindly provided by Dr. R. Monnat, University of Washington). Endogenous WRN in extracts of LCLs (500 μg of total protein) was precipitated with a rabbit polyclonal antibody raised against full-length WRN (23). The immunoprecipitation reactions were carried out as reported (17); WRN-containing immune precipitates were assayed for enzymatic activities.

Assays

Helicase/Exonuclease—DNA helicase/exonuclease activity was detected by the displacement and degradation of a 5′-radiolabeled 20-mer oligonucleotide from a partial duplex with a 46-mer template, as described previously (6). Immune precipitates containing wild-type WRN were routinely used at a dilution of 1:10–1:100; unwinding and degradation were linear in this range. The dilution factor was reduced, as indicated in the figure legends, when assaying variant proteins that diminished WRN activity. Reaction products were resolved by electrophoresis through 12% non-denaturing polyacrylamide gels; the extents of unwinding/degradation were quantified by PhosphorImager (Amersham Biosciences) analysis.

The substrate utilized in our assays affords us the advantage of examining simultaneous unwinding and degradation of DNA; this substrate has been used extensively in our laboratory (6, 7, 17, 23, 24). The sequence of the 20-mer is such that its degradation products migrate anomalously in native polyacrylamide gels. Thus, the lower band represents the unwound 20-mer, whereas the upper band represents exonucleolytic products of the unwound 20-mer. This was independently confirmed from the positions of migration of heat-denatured 19/46 and 20/46 DNA substrates, wherein the 19-mer and 20-mer were 5′-radiolabeled (Fig. 1*B*). Because the helicase and exonuclease work coordinately (25), both bands were included in the quantitation of helicase/exonuclease activity. Activity was quantified as the ratio of products to substrate plus products in each lane.

ATPase Activity—DNA-dependent ATPase activity was measured by assaying radiolabeled phosphate released from [γ-^{32}P]ATP in the presence of single-stranded DNA (6). Free phosphate was complexed by the addition of ammonium molybdate; the phosphomolybdenum complex was extracted with water-saturated *n*-butanol. Radioactivity, in aliquots of the organic phase, was monitored by liquid scintillation counting.

Screening Human DNA Samples for WRN R834C—The amino acid substitution at codon 834 is the result of nucleotide substitution C → T at position 2500 in exon 21. DNA samples from 1559 healthy control individuals were genotyped to estimate the allele frequencies of R834C in various populations. The normal population included 459 Spanish individuals (*i.e.* from Spain, not American Hispanic), 749 Europeans of other ancestries, and 351 African-Americans.

A genomic DNA PCR amplification/restriction digestion-based assay (26) was used to genotype R834C. The assay is based on the presence of the polymorphic site within the recognition sequence of the restriction enzyme TaqI; uncut PCR product is indicative of the presence of 2500T. A 261-bp fragment of genomic DNA was amplified with *Taq* DNA polymerase (Roche Applied Science) using the forward primer 5′-d(TCACCAGTCCTTAATCTGTAAATCAGG)-3′ and the reverse primer 5′-d(AGGAGCACCGTAATGAATGACTTGTC)-3′. Because 2500 C → T does not alter a restriction cleavage site, the reverse primer used for PCR amplification was engineered with a C → A change at bp 2502 to generate the recognition sequence of the enzyme TaqI in the wild-type 2500C allele. Aliquots of the PCR products were digested with TaqI (New England Biolabs) at 65 °C for 2 h; cut and uncut products were resolved by electrophoresis in 2% agarose gels. DNA in uncut PCR products was sequenced to verify the presence of the polymorphism.

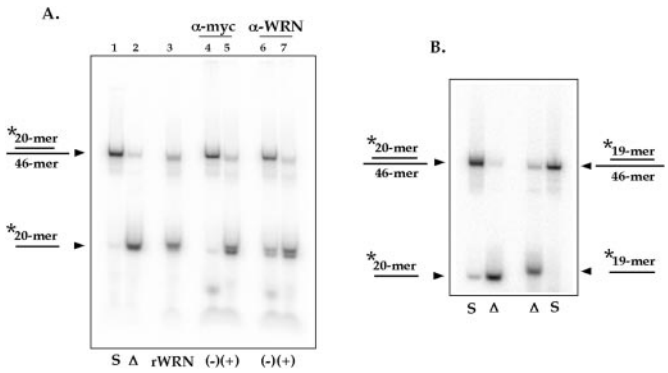

FIG. 1. *A*, specificity of immunoprecipitation with α-myc antibody. WRN in lysates of 293T cells transfected with (+) or without (−) plasmid DNA encoding myc-WRN was immunoprecipitated with either α-myc monoclonal antibody (*lanes 4* and *5*) or α-WRN polyclonal antibody (*lanes 6* and *7*). The immune precipitates were assayed for helicase/exonuclease activity as described. Aliquots of each reaction were electrophoresed through a 12% non-denaturing polyacrylamide gel. The gel was dried and developed by the PhosphorImager imaging device. *Lane 1*: *S*, substrate, 20-mer/46-mer; *lane 2*: Δ, heat-denatured substrate; and *lane 3*: *rWRN*, purified recombinant WRN protein (23). *B*, mobility of DNA substrates in native gels. Two DNA substrates, 19-mer/46-mer and 20-mer/46-mer, prepared by hybridizing radiolabeled 19-mer or 20-mer to a 46-mer template, were electrophoresed through a 12% non-denaturing polyacrylamide gel. *Lanes* labeled "Δ" represent partial duplexes that were heat-denatured prior to electrophoresis.

RESULTS AND DISCUSSION

Polymorphisms are ubiquitous in the human genome, but the effects of only a few on protein function have been determined. Given the positions of the polymorphic sites within the coding sequence of *WRN*, we hypothesized that some of these might affect the enzymatic activities of WRN.

Expression of WRN in 293T Cells—We transiently expressed myc epitope-tagged WRN in mammalian cells, immunoprecipitated, and assayed for helicase and exonuclease activities (Fig. 1*A*). As shown in *lanes 4* and *5*, antibody against the myc epitope selectively precipitated plasmid-encoded WRN, eliminating interference from endogenous WRN. In contrast, immunoprecipitation with a polyclonal WRN antibody resulted in precipitation of activity encoded by both endogenous and transiently expressed WRN (*lanes 6* and *7*). Therefore, all immunoprecipitation reactions with transfected cell lysates were carried out with antibody against the myc-epitope tag on WRN. Plasmid-encoded WRN levels were 50- to 100-fold higher than the level of endogenous WRN, estimated by PhosphorImager analysis of activity assays in at least five independent transfections (data not shown).

Helicase/Exonuclease Activities of Variant Proteins—The six polymorphic sites we analyzed occur throughout the *WRN* coding sequence (Fig. 2*A*) and include both common and infrequent alleles. The activities of each variant protein were assayed at least in duplicate from a minimum of two independent transfections.

F1074L and C1367R—F1074L is a common polymorphism (frequency 0.41) located in the vicinity of the RecQ C-terminal domain (3, 27), whereas C1367R (frequency 0.16), is in close proximity to the nuclear localization signal (28). Both variant proteins exhibited little change (less than 2-fold) in their helicase/exonuclease activities relative to wild-type WRN (Fig. 2, *B* and *D*). Quantitative Western blot analyses of the immunoprecipitated samples revealed that the levels of both proteins were similar to that of transfected wild-type WRN (Fig. 2, *C* and *D*). Beneficial effects of the less common alleles at both these sites have been suggested based upon an association of 1074L with age in Finnish and Mexican populations (19) and an association of 1367R with lower risk of myocardial infarction in the Japanese population (21).

Q724L, S1079L, and T172P—Unlike F1074L and C1367R, Q724L, S1079L, and T172P are infrequent polymorphisms with allele frequencies between 0.01 and 0.02. Q724L and S1079L are located in the helicase domain and in the vicinity of the RecQ C-terminal domain, respectively. Apparent ~2-fold diminution of the enzymatic activities of Q724L and S1079L (Fig. 2, *B* and *D*) was paralleled by a similar -fold reduction in protein levels (Fig. 2, *C* and *D*) such that the specific activities of these variants were not significantly altered relative to control WRN. In fact, alignment of the helicase motifs of RecQ family helicases shows that *E. coli* RecQ and *S. pombe* Rqh1 have leucine at the position corresponding to Gln-724 (29) suggesting that the leucine substitution should not significantly alter catalytic activity.

The helicase/exonuclease activity of T172P was reduced 5-fold relative to that of the control (Fig. 2, *B* and *D*), whereas its level in the immune precipitate was reduced less than 2-fold (Figs. 2, *C* and *D*). Thus, the specific activity of T172P was about 3-fold lower than the control. Interestingly, in addition to the overall decrease in activity, the ratio between the 19-mer product, representing both unwinding and degradation, and the 20-mer, representing unwinding alone, was altered by the T172P substitution (compare *lanes F1074L* in Figs. 2*B* and 4*A* and *lanes T172P* in Fig. 2*B*). The relative preponderance of the unwound 20-mer suggests that T172P may also be defective in degrading DNA and/or coupling degradation to unwinding. This is plausible because Thr-172 maps to the exonuclease domain (Fig. 2*A*). Although a 3-fold reduction in enzymatic activity is a small effect and may not be causal for the development of WS, this polymorphism could affect cellular phenotypes in specific genetic backgrounds and from as yet unappreciated functions of WRN. A heterozygous allele frequency of 0.02 represents a large number of individuals.

R834C—R834C is also an infrequent polymorphism located in the central helicase core of WRN. Alignment of the helicase domains of RecQ helicases from bacteria, yeast, plants, mouse, and humans shows that arginine at the position equivalent to Arg-834 in WRN is invariant (30), implying that it is important for function. We genotyped more than 1500 DNA samples obtained from anonymous donors of known ethnic origins for this polymorphism. R834C was identified in 7 of 459 persons of Spanish ancestry (Fig. 3), with 6 heterozygotes and 1 homozygote being verified by DNA sequencing (not shown). The heterozygous frequency of the 834C allele in this population sample of Spanish ancestry was ~0.007. Interestingly, the R834C variant was not found in 749 individuals of various other European ancestries nor among 351 African-Americans.

Effects of R834C on Helicase/Exonuclease Activities—R834C WRN exhibited a dramatic and reproducible reduction of both helicase and exonuclease activities compared with wild type WRN (Fig. 2, *B* and *D*). Five independent transfections and multiple measurements of activity yielded essentially identical results. Only trace amounts of unwinding/degradation were evident even when amounts in excess of 100-fold that of wild-type WRN were used. Quantification, by serial dilution of immune precipitates containing R834C WRN, revealed that the activity was reduced ~40–50-fold relative to the control, F1074L (Fig. 4*A*). To ensure that there was no degradation of the substrate independent of unwinding, we electrophoresed reaction aliquots displayed in Fig. 4*A* through a 14% urea-polyacrylamide gel (Fig. 4*B*). As observed and quantified, there was a good correlation in the -fold reduction of exonuclease calculated from the native gel (top band of doublet) and the

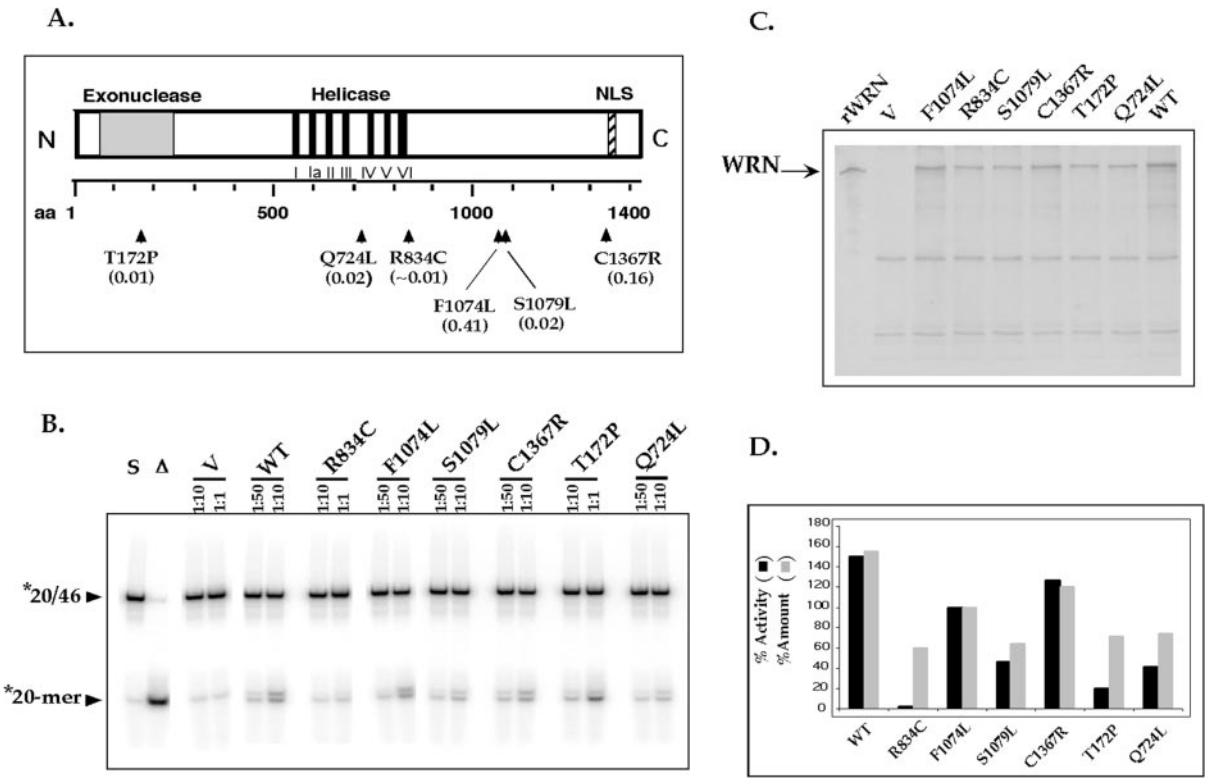

FIG. 2. *A*, location of coding polymorphisms. Schematic of the WRN coding sequence showing positions of the polymorphic amino acid alterations analyzed; the allele frequencies are noted in *parentheses*. The frequencies of F1074L and C1367R are the average allele frequencies calculated from two independent studies (egp.gs.washington.edu) (18). The exonuclease and helicase domains, as well as the nuclear localization signal (*NLS*) are also indicated. *B*, helicase/exonuclease activities of transfected variant WRN proteins. Eight plasmid DNA constructs, including vector lacking WRN (V), or vector-expressing myc-wild-type WRN or each of the six myc-variant WRN proteins, were transfected into 293T cells and precipitated with α-myc monoclonal antibody. The immune precipitates were assayed for helicase/exonuclease activity at dilutions of 1:50 and 1:10, except those with vector, R834C, or T172P that were assayed undiluted or at a 1:10 dilution, as indicated. *S*, substrate, 20-mer/46-mer; Δ, heat-denatured substrate. *C*, Western analysis of immune precipitates. One 1/15th of each sample was electrophoresed in an 8.5% SDS-polyacrylamide gel. The gel was transferred to a polyvinylidene difluoride membrane, blocked overnight with 5% dry milk, and incubated with primary anti-WRN monoclonal antibody (BD Biosciences) and secondary horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham Biosciences). The blot was developed with the ECL Plus reagent (Amersham Biosciences); bands were visualized and quantified by PhosphorImager analysis. The *two prominent bands* other than WRN are the immunoglobulin heavy and light chains. *Lane 1*, positive control: purified, recombinant WRN, is indicated by the *arrow*. *D*, quantification of activity and amounts of variant proteins. The extents of unwinding/degradation (*B*) and the levels of WRN in the immune precipitates (*C*) were quantified by PhosphorImager analyses and normalized to that of F1074L, assigned a value of 100%.

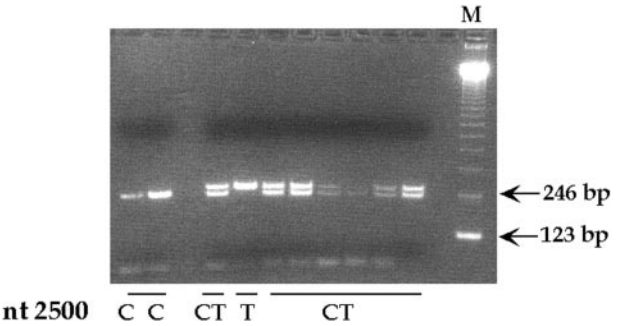

FIG. 3. **Genotypic analysis of R834C.** Genomic DNA from two individuals homozygous for 2500C (Arg-834) (*lanes 1* and *2*), or from one individual homozygous for 2500T (Cys-834) (*lane 5*) or from seven 2500CT heterozygous individuals (*lanes 4* and *6–11*) was PCR-amplified. The PCR products were digested with TaqI; reaction aliquots were electrophoresed through 2% agarose gels. The *lower band* represents the wild-type sequence (2500C), cleaved by the restriction enzyme, whereas the *upper band* is the uncleaved product containing 2500T, verified by direct sequencing. *Lane 4*, positive control of genomic DNA from the R834C heterozygous LCL (Fig. 5; Coriell collection); *lanes 5–11*, DNA from the sampled Spanish population. *M*, 123-bp molecular weight ladder.

denaturing gel, indicating that, at least with this substrate, exonucleolytic degradation is coupled to unwinding.

Because R834C maps to the helicase domain, we monitored its helicase activity independent of exonuclease activity. The helicase was uncoupled from the exonuclease by use of a 3′-end-blocked DNA substrate. The 20-mer oligonucleotide was modified to contain an inverted 3′-3′ link between the terminal and penultimate nucleotides; a partial duplex of the blocked 20-mer/46-mer is refractory to the 3′→5′ exonuclease activity of WRN. By using this substrate, we observed that, as in the case of the unmodified substrate, the helicase activity of R834C WRN was reduced 40- to 50-fold relative to that of the control (Fig. 4*C*).

Two independent plasmid DNA sub-clones containing the R834C substitution showed similar losses of WRN enzymatic activities, eliminating the possibility that a secondary alteration might be responsible for the deficiency. Furthermore, normalizing WRN activity to the activity encoded by a co-transfected LacZ plasmid yielded the same result (not shown), negating the possibility that a difference in transfection efficiency accounted for the major difference in R834C activity. Quantitative immunoblot analyses revealed that, in contrast to the large reduction of enzymatic activity, the level of R834C WRN was reduced less than 2-fold compared with the control (Fig. 2, *C* and *D*). These observations imply that the predominant effect of the R834C substitution is to alter WRN activity rather than protein expression.

Analysis of R834C WRN in Cultured Human LCLs—R834C was identified in DNA from the Polymorphism Discovery Resource data base (egp.gs.washington.edu). To analyze R834C

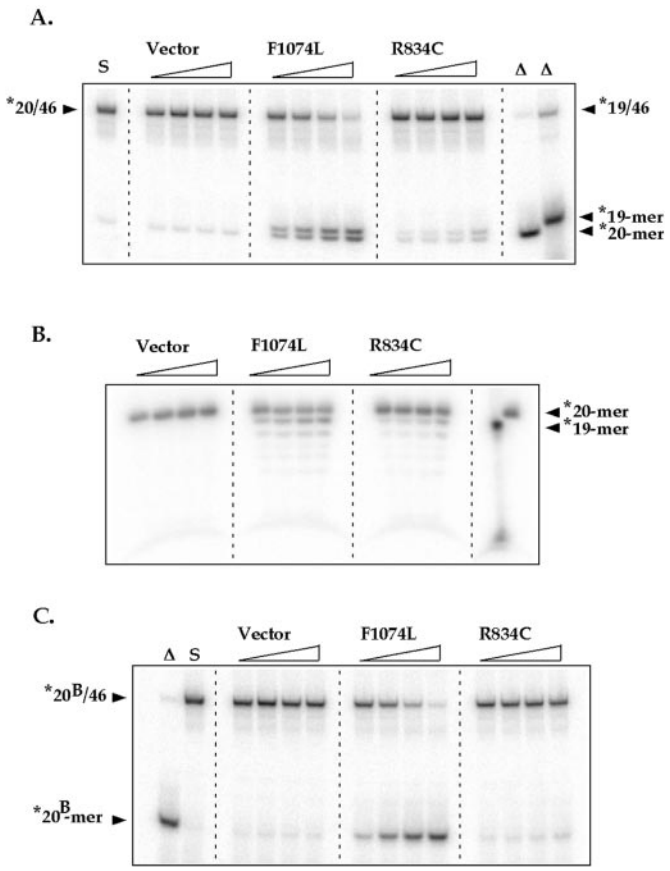

FIG. 4. **Helicase/exonuclease activity of R834C.** The -fold reduction of R834C WRN activity was evaluated by serial dilution of immune precipitates (1:4–1:50). One half of the helicase/exonuclease reaction was electrophoresed through a non-denaturing gel (*A*), as described above. The other half was electrophoresed through a 14% urea-polyacrylamide gel (*B*) to exclusively analyze exonucleolytic products of the reaction. *C*, helicase activity of R834C. The 20-mer oligonucleotide (used above), was modified to contain an inverted 3′-3′ link between the terminal and penultimate nucleotides. The 3′-end blocked 20-mer, radiolabeled at the 5′-end (*$*20^B$*) and hybridized to the 46-mer, was used to monitor helicase activity independent of exonuclease activity. Immune precipitates were diluted, assayed, and analyzed as described.

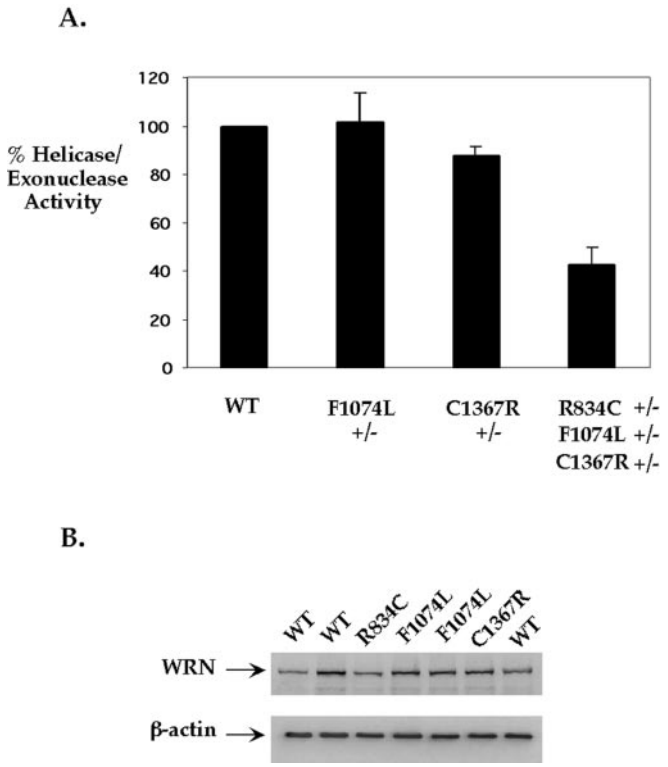

FIG. 5. *A*, helicase/exonuclease activity of endogenous R834C WRN. Exponentially growing Epstein-Barr virus-transformed lymphocytes were lysed and endogenous WRN was immunoprecipitated with α-WRN polyclonal antisera. The helicase/exonuclease activity of immune precipitates was measured as described under "Experimental Procedures." The activities of F1074L, C1367R, and R834C were normalized to that of wild-type WRN. Each *bar* represents multiple measurements from four independent immunoprecipitation assays. Lysates were prepared from three cell lines with wild-type WRN, two cell lines heterozygous for F1074L, and one line each, heterozygous for C1367R WRN or R834C WRN. *B*, quantitative Western blot analysis of endogenous WRN. Levels of WRN in lysates (100 μg each) used for immunoprecipitation were determined by quantitative immunoblot analysis as described in the legend to Fig. 2. The levels were normalized to that of β-actin, an internal loading control.

WRN activity when expressed from its natural promoter, we examined WRN helicase/exonuclease activity in lymphocytic cell lines established from anonymous donors. One individual out of 90 was heterozygous for R834C; this donor was also heterozygous for F1074L and C1367R. Therefore, we compared WRN activity in this cell line to lines derived from six donors, three that had wild-type WRN, two donors that were heterozygous for F1074L WRN, and one individual that was heterozygous for C1367R WRN. We immunoprecipitated endogenous WRN from high salt lysates of lymphocytes with a rabbit polyclonal antibody raised against the full-length protein, and assayed the immune precipitates for helicase/exonuclease activity (Fig. 5*A*). As observed in the transient transfection experiments (Fig. 2), cells heterozygous for F1074L or C1367R had activities essentially similar to those of wild-type WRN. However, multiple measurements with four independently prepared and immunoprecipitated lysates showed that cells that were also heterozygous for R834C had an average 42% of the helicase/exonuclease activity exhibited by controls (Fig. 5*A*). Quantitative Western blot analyses, using β-actin as an internal control, showed that the level of WRN in R834C heterozygous cells was ~90% that of the average WRN level in three wild-type cell lines (Fig. 5*B*). This is consistent with our measurements of R834C WRN levels in transfected cell lysates (Fig. 2, *C* and *D*) and further supports the notion that the R834C polymorphism predominantly affects activity rather than expression of WRN.

Whereas WS cells lack detectable WRN enzymatic activity, cells heterozygous for *WRN* have half as much helicase/exonuclease activity (17). In this respect, cells heterozygous for R834C are similar to *bona fide* WS heterozygous cells in which wild-type *WRN* is the predominant, if not the only, functional allele. An important difference between WS-associated mutations and the R834C polymorphism, however, is that the former are invariably nonsense or frameshift mutations resulting in truncated WRN protein that is undetectable intracellularly (17), whereas R834C is a missense substitution that has minimal effect on WRN levels. R834C represents the first naturally occurring missense amino acid substitution in WRN that markedly decreases helicase and helicase-coupled exonuclease activities.

ATPase Activity of R834C WRN—Because unwinding of DNA is absolutely dependent on ATP (31), we determined if ATP hydrolysis by R834C WRN is diminished. Immune precipitates prepared from cells transfected with vector lacking WRN, or containing F1074L or R834C WRN, were assayed for DNA-dependent ATPase activity, as shown in Fig. 6*A*. Whereas immune precipitates containing F1074L exhibited robust ATP hydrolysis, ATP hydrolysis by R834C WRN was decreased as much as 10-fold. We conclude that the diminished ATPase activ-

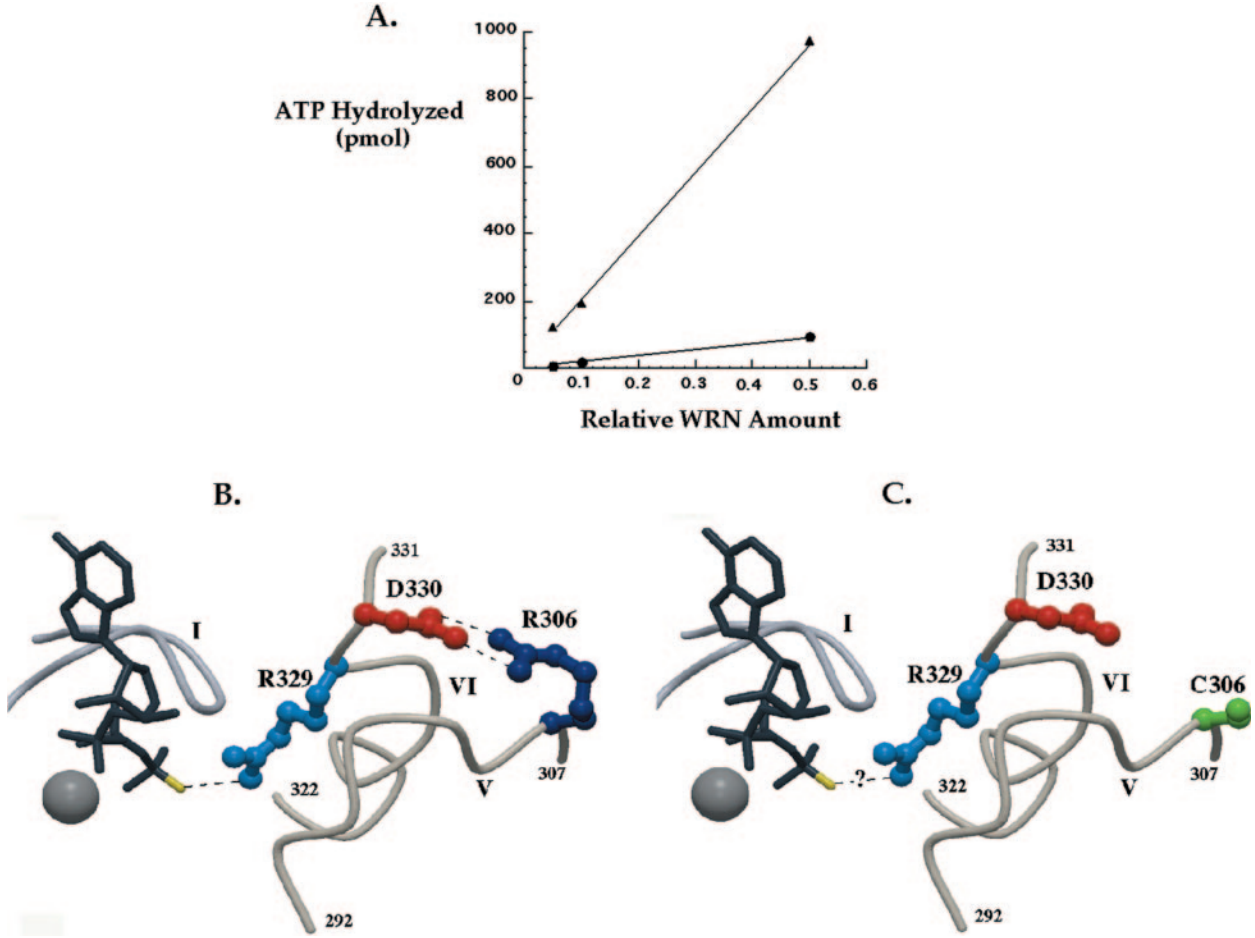

FIG. 6. *A*, ATPase activity of R834C WRN. Immune precipitates of 293T cells transfected with vector lacking WRN or containing F1074L (*filled triangles*) or R834C (*filled circles*) WRN were assayed for the ability to hydrolyze ATP. Indicated amounts (1:2, 1:10, and 1:20 dilutions of immune precipitates) of each sample were incubated with 0.5 mM [γ-^{32}P]ATP in the presence of single-stranded M13mp2 DNA at 37 °C for 40 min. Reactions were terminated by the addition of 0.2 ml of 0.1 N H_2SO_4/1.5 mM NaH_2PO_4, and free radiolabeled phosphate was measured. Nonspecific ATP hydrolysis in samples of vector-transfected cells was subtracted. Data are representative of multiple measurements from six independent experiments. *B* and *C*, model of *E. coli* RecQ helicase bound to ATPγS. The figures show the ATP analog, ATPγS bound in the *E. coli* RecQ helicase catalytic core structure, with domain one on the *left*, showing only the consensus helicase motif I, and domain two on the *right*, showing only helicase motifs V and VI. The *large sphere* is the Mn^{2+} bound to ATPγS; the ATP analog is shown as a *stick figure* with the sulfur atom in *yellow*. Residues likely to be disrupted by the Arg to Cys substitution are shown with *residue coloring*: Arg-306, *dark blue*; Cys-306, *green*; Asp-330, *red*; Arg-329, *light blue*. Arg-329 is closely packed between domains one and two, and is the only residue within domain two that is in hydrogen bonding distance to ATPγS. The *dashed lines* in *B* show hydrogen bond interactions between Arg-306 and Asp-330, and between Arg-329 and ATPγS. The model in *C* suggests that when the hydrogen bond interactions are disrupted by the Cys substitution at amino acid 306, the interaction between Arg-329 and ATPγS may be perturbed (indicated by "*?*"). Coordinates for the RecQ helicase core, PDB code 1OYY (30), were obtained from the Protein Data Bank (33). Mutations were made using the program O (34). The drawing was made using MOLSCRIPT and Raster3D (35, 36).

ity conferred by the R834C substitution could account, at least in part, for the diminished helicase activity of this variant protein.

A recently published high resolution structure of the prototypical *E. coli* RecQ helicase core (30) has revealed that the helicase region comprises two distinct domains separated by a deep cleft. Conserved helicase motifs line the walls of the cleft implying the importance of these motifs in DNA unwinding. Further, a co-crystal structure of RecQ and ATPγS has enabled the identification of the ATP binding site. Based on the published structure, we deduced the following (Fig. 6*B*): (i) Arg-306 (corresponding to Arg-834 in WRN) forms two side chain hydrogen bonds with Asp-330; (ii) Asp-330 has backbone contacts with Arg-329, closely packed between the two domains and the only residue in domain 2 that is in close enough proximity to ATPγS to permit hydrogen bonding with it, and (iii) the carbonyl oxygen of Gly-325 forms a hydrogen bond with the side chain of Arg-329 to further position it near the ATP. Arg-306, Asp-330, and Arg-329 are invariant among 65 bacterial RecQ helicases and are conserved in WRN (30), suggesting that they are critical to helicase function. Substitution of Arg-306 with cysteine (as occurs in WRN R834C) would disrupt the hydrogen bond interactions with Asp-330 (Fig. 6*C*). When the interactions between Arg-306 and Asp-330 are disabled, Arg-329 may be no longer oriented appropriately to interact with, and facilitate hydrolysis of ATP. Thus, by analogy to *E. coli* RecQ, substitution of Arg-834 in human WRN with cysteine could interfere with ATP hydrolysis, an absolute requirement for helicase activity. In fact, this is what we observed (Fig. 6*A*).

As observed with WS, most of the mutations associated with Bloom syndrome truncate the protein and result in lack of detectable BLM protein (29). However, unlike WS, at least six missense substitutions have been linked to Bloom syndrome (29). Four of these map to the helicase domain, and three were shown to abolish both helicase and ATPase activities (32), suggesting that defects in these functions may be primarily responsible for conferring Bloom syndrome phenotypes. The analogy with Bloom syndrome supports the possibility that individuals who are homozygous for the WRN 834C allele could exhibit some of the phenotypes associated with Werner syndrome, including an elevated risk of unusual cancers and early

onset of atherosclerosis, diabetes mellitus, bilateral cataracts, and osteoporosis.

In summary, we report that an infrequent polymorphism that encodes an R834C substitution in the WS protein results in marked diminution of helicase and helicase-coupled exonuclease activity without affecting protein expression. Diminution of activity was established by immunoprecipitation of R834C WRN from transiently transfected mammalian cells and from lymphoblast cells heterozygous for this allele. We inferred, from the structure of the *E. coli* RecQ helicase core, that the Arg to Cys substitution could interfere with ATP binding and thus, ATP hydrolysis. In fact, we present evidence to support this. Future studies should help determine whether the decrease in the enzymatic activities of R834C WRN can be correlated with the occurrence of specific phenotypes, some of which could overlap with those associated with WS, namely, genomic instability or accelerated aging.

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Note Added in Proof—Since the submission of this manuscript, Bohr *et al.* [Bohr, V. A., Metter, E. J., Harrigan, J. A., von Kobbe, C., Liu, J. L., Gray, M. D., Majumdar, A., Wilson, D. M., III, and Seidman, M. M. (2004) *Mech. Ageing Dev.* **125,** 491–496] have also reported that the polymorphism, C13637, does not alter the helicase or exonuclease activities of WRN.

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