WRN helicase expression in Werner syndrome cell lines

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ABSTRACT

Mutations in the chromosome 8p WRN gene cause Werner syndrome (WRN), a human autosomal recessive disease that mimics premature aging and is associated with genetic instability and an increased risk of cancer. All of the WRN mutations identified in WRN patients are predicted to truncate the WRN protein with loss of a C-terminal nuclear localization signal. However, many of these truncated proteins would retain WRN helicase and/or nuclease functional domains. We have used a combination of immune blot and immune precipitation assays to quantify WRN protein and its associated 3′→5′ helicase activity in genetically characterized WRN patient cell lines. None of the cell lines from patients harboring four different WRN mutations contained detectable WRN protein or immune-precipitable WRN helicase activity. Cell lines from WRN heterozygous individuals contained reduced amounts of both WRN protein and helicase activity. Quantitative immune blot analyses indicate that both lymphoblastoid cell lines and fibroblasts contain ~6×10^4 WRN molecules/cell. Our results indicate that most WRN mutations result in functionally equivalent null alleles, that WRN heterozygote effects may result from haploinsufficiency and that successful modeling of WRN pathogenesis in the mouse or in other model systems will require the use of WRN mutations that eliminate WRN protein expression.

INTRODUCTION

Werner syndrome (WRN; MIM #277700) is an uncommon autosomal recessive disease that results from mutational inactivation of the human RecQ family helicase encoded by the chromosome 8p WRN gene (1,2). The Werner phenotype resembles premature aging: after puberty, patients rapidly develop premature graying and loss of hair, scleroderma-like skin changes, osteoporosis, atherosclerosis, bilateral cataract formation, diabetes mellitus and hypogonadism (3,4). Spontaneous genetic instability has been identified in Werner patients, and may be an important determinant of the increased cancer risk in patients (reviewed in 2,5). This increased cancer risk is largely restricted to five tumor types: soft tissue sarcomas, osteosarcoma, thyroid carcinoma, acral lentigenous melanoma and meningioma (3,6). Most Werner patients die prematurely of cancer or cardiovascular disease, with an average age at death of 47 years (4).

The WRN gene, located at 8p11-12, encodes a human RecQ family helicase that has both 3′→5′ helicase and exonuclease activities (7–10). WRN is one of five human RecQ helicases, and one of three that has been associated with a heritable human disease. BLM mutations that reduce or eliminate BLM RecQ helicase activity have been identified in Bloom syndrome patients (11), and a subset of patients with Rothmund–Thomson syndrome have truncating mutations in the human RECQL4 gene (12).

All of the mutations thus far identified in WRN patients are predicted to truncate the WRN protein with a loss of up to 1256 amino acid residues, including the C-terminal nuclear localization signal (NLS), from the 1432 residue native protein (reviewed in 2). We have used a combination of immune blot and immune precipitation assays to detect and quantify WRN protein and WRN helicase activity in cell lines from genetically characterized Werner patients, heterozygotes and controls. Cell lines from patients harboring four different WRN mutations contained no detectable WRN protein or WRN helicase activity; heterozygous individuals had reduced levels of both WRN protein and helicase activity. These results have implications for Werner syndrome pathogenesis, the mechanistic basis for WRN heterozygote effects and the modeling of WRN pathogenesis in murine and other model organisms.

MATERIALS AND METHODS

Cell culture

The Epstein–Barr virus (EBV) transformed B-lymphoblastoid cell lines (LCLs) from Werner pedigrees and the primary and SV40-transformed WRN and control fibroblasts used in this study are detailed in Table 1 and have been previously described (13,14). The mouse hybridoma cell line 9E10 that produces a monoclonal antibody directed against the myc
epitope tag has been previously described (15). Suspension cells were grown in RPMI 1640 medium, and attached cells in Dulbecco’s modified minimal essential medium (DMEM) containing 4.5 g/l glucose. Both media were supplemented with 10% fetal calf serum (Hyclone), 100 U/ml penicillin G sulfate and 100 μg/ml streptomycin sulfate. All cultures were grown in a humidified, 5% CO₂ incubator at 37°C.

Plasmids and transfection

A cDNA encoding full-length WRN was inserted in-frame with the N-terminal myc epitope tag of plasmid pCS2+MT (16) to create plasmid pMM229. This plasmid was used as a substrate for oligonucleotide-directed mutagenesis (Transformer system, Clontech) to generate plasmid pMM247 containing a c.3724C>T mutation—and a resulting new Q1165X termination codon—in the WRN open reading frame. Plasmid pMM248, encoding a myc epitope-tagged K577M WRN protein, was generated by replacing an internal fragment of pMM229 with a comparable WRN cDNA fragment containing a c.1961A>T substitution (7). Plasmid DNAs were transfected into the SV40-transformed WRN fibroblast cell line AG11395 (WS780) by using SuperFect (Qiagen).

Antisera and antibody generation

Rabbit polyclonal antisera SAM1 and SAM2 were raised against recombinant tagged WRN protein. The protein expression vector consisted of pSETB (Invitrogen), into which a WRN cDNA fragment encoding protein residues 722–1432 was inserted in-frame with an N-terminal hexahistidine tag. Recombinant protein expressed in Escherichia coli cells, were lysed by heating for 3 min at 100°C. Lysate proteins were separated by electrophoresis through 6% polyacrylamide–SDS gels, and then transferred onto PVDF membrane by electroblotting (90 min at 100 V in Tris–glycine buffer containing 20% methanol; 19). Non-specific binding sites on membranes were blocked with TBS-T buffer (25 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.1% Tween-20) containing 10% non-fat dry milk (NFDM) prior to the addition of SAM1 or SAM2 IgG (1:1000 dilution) and enhanced Luminol reagent (NEN Life Sciences). The chemiluminescence signal was recorded on BioMAX MR film (Kodak). WRN protein copy number determinations were performed in triplicate, using extracts from 0.5–1.5 × 10⁷ cells and purified recombinant full-length WRN protein of known concentration as a standard (10). Immune blot films were scanned and converted to TIFF files using Photoshop 4.01 (Adobe) prior to quantification using NIH Image (Wayne Rasband, NIH). Experiments in which recombinant WRN protein standards fit a linear equation were used to quantify WRN in cell extracts.

Immune precipitation and helicase activity assays

Frozen cells (~10⁶; wet vol 0.1 ml) were resuspended in 4 vol of ice-cold extraction buffer (20 mM Tris–HCl, pH 8.0, 0.5 M NaCl, 1 mM EDTA, 0.5 mM DTT, 0.5% NP-40, 25% glycerol, 0.2 mM PMSF and 10 μg/ml each of aprotonin, pepstatin and leupeptin), then incubated on ice for 20 min prior to disruption in a teflon-glass homogenizer. The homogenate was centrifuged at 20 000 g for 15 min at 4°C. This procedure reproducibly solubilized 50% of the WRN protein contained in cells. An aliquot of the supernatant (1 mg total protein) was pre-cleared by incubation with a 10% (v/v) suspension of Pansorbin (Staphylococcus aureus cells; CalBiochem) in IP buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 25% glycerol and 0.5% NP-40, 0.05% sodium deoxycholate and 0.005% SDS) for 30–60 min on ice. Cells were pelleted at 20 000 g for 5 min. The resulting pre-cleared supernatant was incubated with an excess of 9130J WRN antisera or with pre-immune serum from the same animal for 60 min at 4°C. WRN:IgG complexes were precipitated by the addition of a 10% (v/v) suspension of S. aureus cells and collected by centrifugation as described above. The pellet was washed three times with 0.5 ml of IP buffer and resuspended in 15 μl of 25 mM Tris–HCl buffer, pH 8.0, 0.5 mM EDTA, 1 mM DTT, 0.05% NP-40 and 25% glycerol. The suspension was assayed immediately for helicase activity, detected by the displacement of a 32P-labeled 20mer oligonucleotide from a 20mer/46mer partial DNA duplex as previously described (7). Helicase assays in which activity was compared among patient, heterozygote and control immune precipitates were performed in the linear range of the unwinding assay (<10% of substrate consumed).

RESULTS

WRN polyclonal antisera

The ability of three newly generated rabbit polyclonal antisera to detect WRN was determined by immune blot analyses of cell extracts prepared by transfecting WRN cell line AG11395 with a WRN cDNA expression vector that encoded myc epitope-tagged full-length WRN or a Q1165X truncated WRN protein. The Q1165X protein was generated from a WRN cDNA expression vector that encoded a myc epitope tag of plasmid pCS2+MT (16) by incubation with a 10% (v/v) suspension of Pansorbin (Staphylococcus aureus cells; CalBiochem) in IP buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 25% glycerol and 0.5% NP-40, 0.05% sodium deoxycholate and 0.005% SDS) for 30–60 min on ice. Cells were pelleted at 20 000 g for 5 min. The resulting pre-cleared supernatant was incubated with an excess of 9130J WRN antisera or with pre-immune serum from the same animal for 60 min at 4°C. WRN:IgG complexes were precipitated by the addition of a 10% (v/v) suspension of S. aureus cells and collected by centrifugation as described above. The pellet was washed three times with 0.5 ml of IP buffer and resuspended in 15 μl of 25 mM Tris–HCl buffer, pH 8.0, 0.5 mM EDTA, 1 mM DTT, 0.05% NP-40 and 25% glycerol. The suspension was assayed immediately for helicase activity, detected by the displacement of a 32P-labeled 20mer oligonucleotide from a 20mer/46mer partial DNA duplex as previously described (7). Helicase assays in which activity was compared among patient, heterozygote and control immune precipitates were performed in the linear range of the unwinding assay (<10% of substrate consumed).
Figure 1. WRN antisera detect full-length and truncated WRN proteins. (A) IgG fractions of two rabbit polyclonal antisera, 9130J (top) raised against full-length WRN, and SAM1 (bottom) raised against the C-terminal half of WRN, were used in immune blot analyses to detect three myc epitope-tagged proteins. Each lane contains equivalent amounts of total cell extract from AG11395 WRN fibroblasts expressing full-length WRN (mWT; 174 kDa), truncated Q1165X WRN (mQ1165X; 144 kDa) or control bacterial β-galactosidase (mGalZ; 128 kDa). The myc epitope comprises 11.7 kDa of each tagged protein. WRN proteins are detected by each antiWRN IgG fraction, though neither detected the β-galactosidase control. (B) Immune blots shown in (A) were dried to inactivate the peroxidase-conjugated secondary antibody, then rehydrated and incubated with 9E10 monoclonal antibody against the myc epitope tag of each protein. This allows the relative abundance of each protein to be determined, and the comparative sensitivity of detection of the antiWRN IgG fractions to be estimated (see Results).

Figure 2. WRN protein is not detected in WRN patient cell lines. (A) Whole cell extracts prepared from 10^6 control (WRN +/+; TUR90050) cells (lane 1) or from 2 × 10^6 Werner patient (WRN −/−) cells (lanes 2–5) containing four different WRN mutations (Table 1) were used for immune blot analyses. WRN protein was detected only in the WRN +/+ control (lane 1). Additional cross-reacting bands of lower or higher molecular weight do not appear to be WRN-derived: they are present whether WRN protein is detected or absent, and do not differ between cell lines carrying different WRN mutations. (B) Full-length and truncated WRN proteins have comparable stability in WRN patient cells or cell extracts. Extracts from 2 × 10^6 WRN patient (WRN −/−; TUR900010) cells lacking detectable Q1165X WRN protein (lane 1) was mixed with extracts from: 10^6 transfected AG11395 WRN fibroblasts expressing myc epitope-tagged recombinant Q1165X protein (lane 2, mQ1165X); 10^6 WRN +/+ cells from a within-pedigree control (TUR90050, lane 3; +WT); or extract from 10^6 transfected AG11395 WRN fibroblasts expressing myc epitope-tagged recombinant full-length WRN protein (lane 4, +mWT) prior to immune blot analysis. The position of full-length native WRN is indicated by the open arrowhead in both panels. The SAM1 antiWRN IgG fraction was used for both analyses.

by WRN antisera (Fig. 1B; compare band intensities with 1A). SAM1 IgG was the most sensitive of the three WRN IgG fractions: it was able to detect as little as 40 pg (~2.5 × 10^6 molecules) of purified recombinant WRN.

Immune blot analyses

We used the most sensitive of the WRN antisera, SAM1 IgG, in immune blot analyses to search for WRN protein in cell line extracts from Werner patients carrying four different WRN mutations. The lengths of the predicted WRN proteins encoded by these mutant alleles ranged from 368 to 1245 amino acids (Table 1). Three of the four predicted proteins should be readily detected by the SAM1 IgG fraction, as they share epitopes with the C-terminal half of WRN that was used to raise the SAM1 antiserum. However, the SAM1 IgG did not detect the predicted WRN proteins in extracts from any of the Werner patient cell lines we studied (Fig. 2A, lanes 2–5). Immune blot analyses using 9130J IgG raised against full-length WRN also failed to detect the predicted R369X WRN protein in patient cells from the LGS Werner pedigree (Table 1; additional data not shown).

We were able to detect full-length WRN protein in reduced amount in cell lines derived from WRN heterozygotes in all four pedigrees. A detection sensitivity of ≥2% was established by determining the number of control (WRN +/+ ) cells that, when added to 2 × 10^6 Werner patient cells, allowed the consistent detection of WRN in immune blot analyses. This was 4 × 10^6 cells, or 2% of the cell number, included in immune blot analyses (Fig. 2B, lanes 1 and 3; additional results not shown). A comparable sensitivity of detection for truncated WRN was established using extracts from AG11395 WRN fibroblasts that had been transiently transfected with plasmids encoding myc epitope-tagged Q1165X or full-length WRN protein (Fig. 2B, lanes 2 and 4; additional results not shown). A majority of the recombinant protein expressed in patient cells consisted of the predicted full-length native or truncated WRN protein (Figs 2B and 4A). Low molecular weight, cross-reacting bands detected in extracts from transfected cells may represent degradation products of full-length or Q1165X WRN protein (Fig. 2B, lowest bands in lanes 2 and 4).

Immune precipitation analyses

We performed helicase activity assays on immune precipitates to detect and quantify WRN helicase activity in cell extracts. In three of the four Werner pedigrees analyzed, mutant WRN alleles are predicted to encode truncated proteins that retain both the WRN helicase and N-terminal nuclease functional domains (ZM, TUR and SYR pedigrees; Table 1). However, we could not detect helicase activity in immune precipitates from any of the four Werner pedigrees studied (Fig. 3). Pre-immune serum did not precipitate detectable activity from
WRN-positive extracts, and extracts from heterozygotes having one intact WRN allele had consistently reduced levels of helicase activity when compared with within-pedigree controls (Fig. 3; additional data not shown).

Table 1. Cells and cellular WRN content as a function of WRN genotype

<table>
<thead>
<tr>
<th>Cell line/strain</th>
<th>WRN genotype</th>
<th>mutation</th>
<th>Predicted WRN protein</th>
<th>WRN content (×10^{-15} g/cell)</th>
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<tr>
<td>LGS90610</td>
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<td>c.1336C&gt;T</td>
<td>R369X</td>
<td>not detected</td>
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<td>1246X</td>
<td>not detected</td>
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<td>+/+</td>
<td>none</td>
<td>1432</td>
<td>16.0 ± 7.1</td>
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_a_ Cell lines/strains: AG11395 (also known as WS780) and GM639 are SV40-transformed fibroblast cell lines; HDF88-1 is a primary human diploid fibroblast cell strain. All other cells are Epstein–Barr virus-transformed B-lymphoblastoid cell lines. 

_b_ WRN mutation: mutations in cell lines are given following suggested nomenclature of the Nomenclature Working Group (41), using the WRN cDNA as a numbering reference. Mutations are described in detail at http://www.pathology.washington.edu/werner/, the WRN Locus-Specific Mutation Database. A print version of the database is also available (2).

_c_ Predicted WRN protein: the number indicates remaining amino acid residues in predicted WRN proteins encoded by mutant alleles, and letters indicate the coding change(s) produced by the indicated mutation in single letter amino acid code with ‘X’ signifying a termination codon. The native WRN protein contains 1432 amino acid residues.

_d_ WRN content: the value shown is the mean and standard deviation of at least three independent determinations per cell line. ‘Not detected’ indicates that in comparable assays WRN could not be detected at a sensitivity level of 20.1 × 10^{-15} g/cell.

The K577M WRN protein has a methionine substituted for the catalytically important lysine at residue 577 in the WRN helicase consensus domain I/Walker A box. This substitution has been previously shown to inactivate WRN helicase activity (7,10).

Cellular WRN content

We used quantitative immune blotting versus a purified recombinant WRN protein standard to determine the number of WRN copies in different patient and control cell extracts (Fig. 5). Control LCLs from different Werner pedigrees contained 9.2–22.8 × 10^{-15} g of WRN protein/cell (Table 1). This corresponds to 3.4–8.4 × 10^{10} WRN molecules/cell. The variation in WRN content/cell was <3-fold between unrelated individuals, and heterozygous cell lines contained reduced amounts of WRN protein as compared with within-pedigree controls (Table 1). There was a 50% reduction in protein level in cell lines containing stop codons within a WRN exon (LGS and TUR; Table 1), and a reduction of >50% in cell lines containing mutations that altered splice sites (ZM and SYR; Table 1). The WRN content of SV40-transformed control fibroblast cell line GM639 and the primary human diploid fibroblast strain HDF88-1, ~16 × 10^{-15} g/cell, was comparable to that observed in control LCLs (Table 1).

DISCUSSION

We have analyzed WRN helicase expression in genetically characterized WRN patient, heterozygote and control cell lines by immune blot and immune precipitation-helicase activity assays. The cell lines we used contained four different, mutant WRN alleles that encode predicted WRN proteins of 368 to
1245 amino acid residues (Table 1). None of these predicted proteins could be detected in immune blot or immune precipitation/helicase activity assays. A recent study corroborates the results of our immune blot analyses of cell lines from the TUR and SYR pedigrees encoding, respectively, Q1165X and 1246X WRN proteins (20). Further, it supports the suggestion that most disease-associated WRN mutations are functionally equivalent null alleles: only an uncommon c.4146–4147insA single base insertion, the most C-terminal equivalent null alleles: only an uncommon c.4146–4147insA single base insertion, the most C-terminal

The absence of detectable WRN protein from patient cells also indicates that the accurate modeling of WRN pathogenesis will require the use of mutations that eliminate or sharply reduce WRN protein expression. A recently published mouse model of Werner syndrome (26) may be problematic in this regard: the targeted alleles containing an in-frame deletion of the murine WRN helicase consensus domain produce a readily detectable truncated WRN protein.

The absence of detectable WRN protein from patient cells indicates that the Werner syndrome develops when both the WRN helicase and nuclease activities are severely reduced. The common biochemical phenotype of different WRN mutations also indicates that clinical and pathologic differences between WRN patients or patient groups (for example 25) are best explained by genetic background or environmental exposure differences, rather than the expression of different, mutant WRN alleles.

Several heterozygote effects have been observed in cells and cell lines from WRN patients. These include the intermediate sensitivity of WRN heterozygous lymphoblastoid cell lines to the DNA damaging agents 4NQO (27) and camptothecin (28,29), and the presence of genetic instability in vivo in the
erythroid (red blood cell) lineage of WRN heterozygotes (M. Moser et al., unpublished results). The presence of reduced amounts of native WRN and the absence of truncated mutant WRN protein in WRN heterozygote cell lines (Fig. 2 and additional data not shown) indicates that the simplest model for these WRN heterozygote effects—haploinsufficiency—is likely to be correct.

As part of the immune blot analyses of WRN expression in cell lines from WRN pedigrees, we also determined the number of WRN molecules per cell. This was done by performing quantitative immune blot analyses using patient or control cell extracts and purified recombinant WRN protein as a standard. The number of WRN molecules/cell, ~60 000 in control cell extracts and purified recombinant WRN protein as number of WRN molecules per cell. This was done by WRN data not shown) indicates that the simplest model for these proteins (e.g. c-Myc, 30; topoisomerases II

Recent results have begun to suggest functions for WRN in human somatic cells. A role for WRN in DNA repair is suggested by the observation that Werner cells are selectively sensitive to the DNA damaging agents 4-nitroquinoline 1-oxide and camptothecin (27–29,36). A second clue to in vivo function comes from identification of the Xenopus WRN homolog, FFA-1, as a protein required for replication focus formation in Xenopus egg extracts (37). This observation is particularly intriguing, as Werner cells and cell lines display DNA replication and S-phase abnormalities (reviewed in 5). A third clue to function comes from the recent finding that WRN and BLM can unwind G'2 triplet repeat and G4 tetraplex DNAs, respectively (38,39). These observations in concert—of selective sensitivity to DNA damaging agents, S-phase or replication abnormalities and the ability to unwind unusual DNA topologies—suggest that WRN may process unusual structures that are arising during DNA replication, recombination or DNA repair at unusual sites (e.g. triplet repeat or telomeric DNAs; 40). Further biochemical and functional analyses should indicate how a reduction or loss of the WRN helicase and exonuclease activities promotes elevated levels of somatic mutation and the development of both neoplastic and non-neoplastic disease in specific human cell lineages.

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