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Mechanisms of Ageing and Development
122 (2001) 921–944

mechanisms of ageing and development

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Review

Unwinding the molecular basis of the Werner syndrome

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Received 24 January 2001; accepted 26 January 2001

Abstract

Werner syndrome (WS) is an autosomal recessive disease manifested by the premature onset of age-related phenotypes, including diseases such as atherosclerosis and cancer. This mimicry of normal aging with the possible exception of central nervous system manifestations has made it a focus of recent molecular studies on the pathophysiology of aging. In culture, cells obtained from patients with WS are genetically unstable, characterized by an increased frequency of nonclonal translocations and extensive DNA deletions. The WS gene product (WRN) is a DNA helicase belonging to the RecQ family, but is unique within this family in that it also contains an exonuclease activity. In addition to unwinding double-stranded DNA, WRN helicase is able to resolve aberrant DNA structures such as G4 tetraplexes, triplexes and 4-way junctions. Concordant with this structure-specificity, WRN exonuclease preferentially hydrolyzes alternative DNA that contains bubbles, extra-helical loops, 3-way junctions or 4-way junctions. WRN has been shown to bind to and/or functionally interact with other proteins, including replication protein A (RPA), proliferating cell nuclear antigen (PCNA), DNA topoisomerase I, Ku 86/70, DNA polymerase δ and p53. Each of these interacting proteins is involved in DNA transactions including those that resolve alternative DNA structures or repair DNA damage. The biochemical activities of WRN and the functions of WRN associated proteins suggest that in vivo WRN resolves DNA topological or structural aberrations that either occur during DNA metabolic pro-

Abbreviations: WS, Werner syndrome; RPA, Replication protein A ; PCNA, Proliferating cell nuclear antigen.

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PII: S0047-6374(01)00248-2

cesses such as recombination, replication and repair, or are the outcome of DNA damage.

Keywords: Werner syndrome; Replication protein A; Proliferating cell nuclear antigen

1. Introduction

There has been a recent acceleration of investigations on Werner syndrome (WS) since the gene (*WRN*) mutated in this disease was identified in 1996 (Yu et al., 1996 and Fig. 1). The cloning of *WRN* was a landmark in aging research. It extends from 1904 when Otto Werner first described in his dissertation this premature aging syndrome (Werner, 1904) and builds on the extensive studies of George Martin and colleagues that established the biological and cytological alterations of WS cells (Martin et al., 1999). WS is classified as adult progeria distinct from childhood progerias such as Hutchinson–Gilford progeria (Brown et al., 1985). Clinical manifestations of WS include cataracts, atrophy, short stature, premature hair-greying and symptoms of age-related diseases such as type II diabetes mellitus, osteoporosis, soft tissue calcification, premature atherosclerosis and neoplasms of mesenchymal origins (Salk, 1982; Martin et al., 1999). With the exception of central nervous system degeneration, this disease provides a stunning mimicry of the aging phenotype. How can mutations in one gene result in so many age-related manifestations? Will an understanding of the biochemical properties of the WS gene yield significant insight into the overall phenomena of aging?

In culture, fibroblast cells from WS patients display limited replicative potentials (Martin et al., 1970) and a prolonged S-phase (Poot et al., 1992), similar to that exhibited by fibroblasts from older individuals. Analyses of chromosomes from WS cells showed that they contained increased numbers of abnormalities including reciprocal translocations, deletions and inversions (Salk et al., 1981). Inspection at the single gene level has also revealed the scenario of increased mutations, mainly in the form of extensive DNA deletions (Fukuchi et al., 1989). Thus, WS can be classified amongst diseases such as Bloom's syndrome, xeroderma pigmentosum, ataxia telangiectasia and Faconi's anemia that are widely referred as chromosome instability syndromes (Meyn, 1997). While these diseases exhibit an elevated incidence of cancer, they do not exhibit the wide spectrum of aging phenotypes that is a hallmark of WS.

The WS gene, *WRN*, is classified as a RecQ DNA helicase (Yu et al., 1996; Gray et al., 1997; Suzuki et al., 1997) and interestingly, also encodes an exonuclease activity (Huang et al., 1998; Shen et al., 1998a; Suzuki et al., 1999) that differentiates it from other RecQ family members (Oshima, 2000; Shen and Loeb, 2000a). Both activities were established using recombinant proteins purified from the insect cells that are infected with a baculovirus that encoded human *WRN*. WRN functions in vitro as a DNA helicase in that it unwinds double-stranded DNA using ATP as the energy source. The separation of complementary DNA strands is central to DNA replication, recombination and many DNA repair processes.

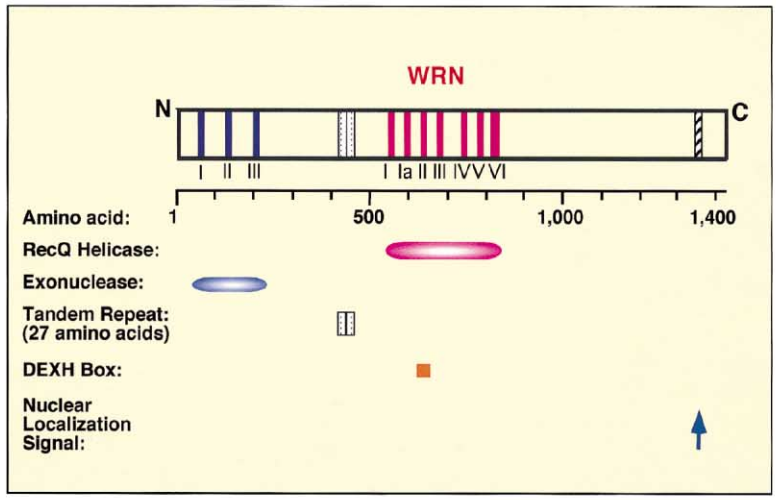

Fig. 1

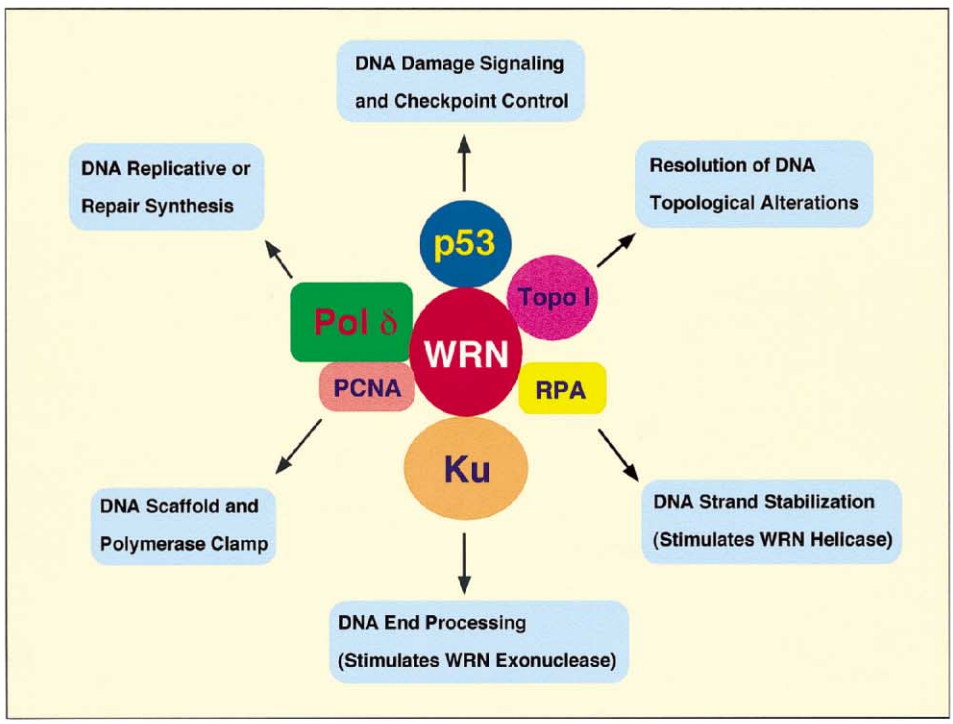

Fig. 2

Fig. 1. Schematic construct of WRN helicase/exonuclease. WRN protein (1432 amino acids) contains a centrally located DEXH helicase domain with the conserved seven motifs that are homologous to the RecQ family of DNA helicases. In addition to the central helicase domain, a conserved three-motif exonuclease domain also resides on the N-terminus. A nuclear localization signal (NLS) is located at the C-terminus (amino acid 1370–1375) and a direct repeat of 27 amino acids is situated between the exonuclease and helicase domains.

Fig. 2. WRN-interacting proteins and their proposed functions. WRN interacts with a variety of proteins such as DNA polymerase δ (Pol δ), proliferating cell nuclear antigen PCNA, replication protein A RPA and DNA topoisomerase I (Topo I) that are mainly involved in DNA replication and repair synthesis. Other interacting proteins such as the human Ku 86/70 complex and the tumor suppressor gene product p53 suggest functions for WRN in DNA repair and/or checkpoint control.

Recent reports indicate that WRN helicase also resolves alternative DNA structures such as G4 tetraplexes (Fry and Loeb, 1999) that may form during processes of DNA metabolism and thus impair these processes. WRN also can drive branch migration of Holliday junctions (Constantinou et al., 2000) and thus might accelerate and facilitate DNA recombination. The exonuclease activity of WRN acts preferentially on alternative DNA structures such as bubble, loop, stem-loop and 3- or 4-way junction DNA (Shen and Loeb, 2000b). In search for a possible WRN function(s) in cells, several proteins that functionally or physically interact with WRN have been identified (Fig. 2), including the Ku 86/70 complex that dramatically enhances the WRN exonuclease activity (Cooper et al., 2000).

Although many insights of WS, particularly at the molecular level, have been recently uncovered, we still lack an understanding of the pathology of this disease. Are both catalytic activities of WRN critical for the WS phenotypes? If so, what is the role of these biochemical activities in DNA metabolic processes? More importantly, how does WRN contribute to maintaining genetic stability and when mutated, lead to a premature aging phenotype, through which process and partnering with which proteins? And finally, how can we relate a single gene (i.e. WRN helicase/exonuclease) malfunction to genetic instability and to human aging? Our goal in constructing this review and in presenting different hypotheses is to stimulate investigations into the role of DNA helicases in cellular metabolism, the pathology of WS and ultimately to understand the role of genetic instability in aging.

2. Werner syndrome and human RecQ helicase-deficient diseases

So far, five human RecQ helicases have been identified and characterized, including RecQL, BLM, WRN, RecQ4 and RecQ5 (see Karow et al., 2000a; Oshima, 2000; Shen and Loeb, 2000a; van Brabant et al., 2000 for reviews). Mutations in three of these genes are associated with genetic diseases, i.e. WRN for Werner syndrome (Yu et al., 1996), BLM for the Bloom syndrome (BS) (Ellis et al., 1995) and RecQ4 for the Rothmund–Thomson syndrome (RTS) (Kitao et al., 1999). Although the clinical manifestations of these autosomal recessive diseases differ from one another, each of them exhibits a predilection for cancer and manifests genetic instability (Shen and Loeb, 2000a; van Brabant et al., 2000). For example, WS patients have increased risk of soft tissue sarcomas, whereas non-Hodgkin's lymphoma and acute lymphocytic leukemia occur frequently in BS patients, and RTS patients have increased incidence of skin cancers and osteosarcomas. The chromosomal manifestations of genetic instability in these diseases are different. Reciprocal chromosomal translocations and extensive genomic deletions characterize WS. In contrast, BS presents a marked increase in rates of sister chromatid exchange that is not characteristic of either WS or RTS. Limited studies on RTS indicate frequent chromosomal rearrangements that result in somatic mosaicism. It is thus compelling to propose that human RecQ helicase plays a role in maintaining genomic integrity, and that defects on RecQ lead to the 'mutator

phenotype' (Loeb, 1991) and are causally associated with the development of neoplasia. However, the pathways that result in genetic instability are likely to be different in these diseases. These diseases are recessive resulting from mutations in both copies of the corresponding RecQ helicase; heterozygotes in the population harboring mutations in a single copy of these genes are much more frequent. The incidence of diseases in the heterozygote populations needs to be carefully analyzed.

How does the WRN helicase (or exonuclease) contribute to the avoidance of genetic aberrations? Analysis of *WRN* mutations in WS patients indicates that both copies of the WS gene are inactivated (Moser et al., 1999). The finding that all WS mutations (nonsense, frame-shift or insertion/deletion) give rise to truncated proteins lacking a putative C-terminal nuclear localization signal (NLS, Matsumoto et al., 1997) supports the hypothesis that loss of WRN function is the cause for WS. It is surprising that no missense mutation has been reported in WS even though some of these would presumably result in loss of WRN activity. In contrast, missense mutations are frequently found in BS (Foucault et al., 1997). The lack of missense mutations in *WRN* may be an important clue to its biological function that so far has evaded explanation.

3. Cellular properties of WRN

3.1. Cellular localization

The WS gene (*WRN*) is located on chromosome 8 at 8p12 (Goto et al., 1992), and encompasses 35 exons (Yu et al., 1996). The *WRN* gene product comprises 1432 amino acids with a molecular weight of about 165 kDa (Yu et al., 1996). The WRN protein is expressed in all tissues so far examined (Yu et al., 1996). In cells, WRN primarily localizes to the nucleolus (Gray et al., 1998; Marciniak et al., 1998), a place where ribosomal RNA is synthesized. However, WRN can also be detected in the nucleoplasm (Shiratori et al., 1999), particularly after treatment of cells with genotoxic agents such as 4-nitroquinoline-1-oxide (4-NQO, Gray et al., 1998) and hydroxyurea (Constantinou et al., 2000). Localization in the nucleolous could indicate a role in the synthesis of ribosomal RNA and mutations in WRN might reduce overall protein synthesis, a hypothesis compatible with aging. However, we lack any evidence for changes in ribosomal gene copy number or preferential rearrangements of ribosomal genes during human aging.

3.2. DNA damage response

Accumulation of unrepaired DNA damage has been frequently proposed as a mechanism for aging. These damages could be responsible for the increase in mutagenesis in aged cells (Martin, 1991) as well as an impediment to gene expression. Evidence for the involvement of WRN in the cellular responses to DNA

damage or in known pathways for DNA repair is limited. Perhaps the strongest evidence is the association with p53, a gene invariably induced as a result of DNA damage leading to apoptosis. Attenuation of p53-mediated apoptosis was reported recently in several WS fibroblast cell lines, and this defect could be restored by expression of wild type WRN (Spillare et al., 1999). Further studies indicate that WS cells exhibit attenuated and delayed induction of p53 after UV-irradiation or exposure to camptothecin (a type I DNA topoisomerase inhibitor) (Blander et al., 2000), suggesting a role for WRN in the activation of p53 in response to certain types of DNA damage. The camptothecin sensitivity should be rather specific since WS cells are not particularly sensitive to other DNA damaging agents such as alkylating agents, hydroxyurea, bleomycin, UV-irradiation and X-ray (see Shen and Loeb, 2000a for review). Not until recently, 4-NQO and camptothecin have been the only documented chemicals that confer hypersensitivity in WS cells (Ogburn et al., 1997; Poot et al., 1999). However, a recent report indicates that WS cells are hypersensitive to a wide range of DNA inter-strand cross-linking agents such as mitomycin (Poot et al., 2001), further suggesting a role for WRN in DNA damage response.

3.3. Maintaining telomere stability

Telomere shortening is a hallmark of the senescence of normal human fibroblasts. As a result, many investigations have considered a role for WRN in the maintenance of telomere length. Telomeres in primary WS fibroblasts tend to shorten faster than those in control cells during sequential passages in culture (Schulz et al., 1996). However, the length of WS telomeres are not significantly shorter than that of controls when WS cells stop dividing, suggesting that telomere shortening is not responsible for accelerated replicative senescence in WS cells (Schulz et al., 1996). These results are difficult to interpret since WS lymphoblastoid lines transformed with Epstein–Barr virus exhibit marked variations in teleomere lengths (Tahara et al., 1997). In contrast to these ambiguous results, recent studies indicate that forced expression of telomerase (hTERT) in WS fibroblasts confers an extended cellular life span and immortality (Wyllie et al., 2000), suggesting that WRN mutations also accelerate normal telomere-driven replicative senescence with a mechanism yet to be established.

4. Lessons from *Escherichia coli* RecQ

The role of RecQ in various aspects of DNA metabolism has been studied extensively in *Escherichia coli*, in which genetics can be so precisely delineated. Even though these studies should give us clues to the function of WRN in human cells, it is important to recognize that WRN contains an exonucleolytic activity that is lacking in RecQ and that *E. coli* do not age.

4.1. A suppressor of illegitimate recombination

E. coli RecQ is the prototype enzyme in the RecQ family of DNA helicases. Historically, it has been considered to function primarily in the RecF pathway of homologous recombination. The implication then is that WRN also functions in homologous recombination. However, *recQ* mutants also exhibit an increased frequency of illegitimate recombination, a process that requires only short sequence homologies to establish DNA strand exchange (Hanada et al., 1997). The later result suggests that RecQ may function as a suppressor of illegitimate recombination.

The genetic evidence for RecQ in suppressing illegitimate recombination was fortified by biochemical studies. RecQ, in concert with RecA and single-stranded DNA binding protein (SSB), can initiate and disrupt DNA recombination in in vitro assays (Harmon and Kowalczykowski, 1998). In accord with the genetic evidence, this result suggests two putative in vivo functions for RecQ, (1) an initiator of homologous recombination and (2) a disrupter of joint molecules formed by illegitimate recombination. These findings also provide an important mechanistic explanation for how RecQ can suppress illegitimate recombination in vivo — by dissociating the recombinational intermediate (or the joint molecule), a DNA structure containing a 3- or 4-way junction.

It is thus not surprising to learn that WRN preferentially binds to 3- or 4-way junction DNA (Shen and Loeb, 2000b) and unwinds Holliday junctions (Constantinou et al., 2000). Will WRN initiate and disrupt joint molecules just like *E. coli* RecQ, or does WRN also function as a suppressor of illegitimate recombination in humans? Further biochemical and genetic characterizations of WRN function(s) are required to address these questions. Nonetheless, genetic studies in *Saccharomyces cerevisiae* have shown that *WRN* can suppress the hyper-recombination phenotype (both homologous and illegitimate) exhibited by a *sgs*1 mutant that lacks the sole endogenous RecQ homolog (Yamagata et al., 1998).

4.2. Involvement in re-initiation of stalled replication forks

The RecF pathway (which includes *recF*, *recO*, *recR*, *recQ* and *recJ*) may play multiple roles in *E. coli* in addition to homologous recombination. RecF and RecR are essential in preventing extensive degradation of newly synthesized DNA at the damage-blocked replication fork following UV-irradiation and also participate in resuming replication (Courcelle et al., 1997). Unlike *recF* and *recR*, *recQ* cells are not hypersensitive to UV, reminiscent of WS cells. However, additional work shows that RecQ together with RecJ, a $5' \rightarrow 3'$ exonuclease that hydrolyzes single-stranded DNA (ssDNA), selectively degrades nascent lagging strand of the disrupted replication fork following UV-irradiation (Courcelle and Hanawalt, 1999). The $3' \rightarrow 5'$ RecQ helicase might play a role in displacing the lagging strand and thus facilitating RecJ to degrade the resulting ssDNA from 5′ to 3′. Therefore, RecQ can also be considered as a replication protein (Kogoma, 1997) with a special function of processing stalled replication forks. Interestingly, WRN, with both the characteris-

tic RecQ helicase activity (Yu et al., 1996; Gray et al., 1997; Suzuki et al., 1997) and an extraordinary $3' \rightarrow 5'$ dsDNA exonuclease activity (Huang et al., 1998; Shen et al., 1998a), is able to bind, unwind and process a synthetic replication fork in vitro (Shen and Loeb, unpublished data). In accord with the WS phenotype of impaired S-phase (Poot et al., 1992), we surmise that WRN might play an important role in processing disrupted forks during DNA replication.

4.3. Involvement in recombinational repair

Recombinational repair is one of the strategies that *E. coli* can use to resume a stalled replication fork. The RecF pathway also plays a role in recombinational repair, particularly in a *recBC sbcBC* background — where RecF replaces the inactive, major recombinational pathway RecBCD (see Kowalczykowski et al., 1994 for review). Working models describing how the recombinational repair machinery deals with a stalled replication fork have been proposed elsewhere (Kuzminov, 1995; Cox, 1997, 1998). The involvement of RecFOR proteins (gene products of *recF*, *recO* and *recR*) in stalled fork-mediated recombination events has been observed, although the role of RecQ in this process has yet to be established. Nonetheless, RecQ along with RecF, RecR and RecJ has been shown to be a key factor in processing a stalled fork (Courcelle and Hanawalt, 1999; Courcelle et al., 1997), and by this means may facilitate further resumption of replication. Since RecQ is able to initiate and disrupt a recombinational event (Harmon and Kowalczykowski, 1998), RecQ may function in recombinational repair by modulating D-loop formation and/or joint molecule resolution.

5. Biochemical properties of WRN

5.1. Helicase

The characteristic $3' \rightarrow 5'$ RecQ helicase activity of WRN has been documented (Gray et al., 1997; Suzuki et al., 1997; Shen et al., 1998b) and summarized in Table 1. Notably, studies delineating DNA substrate specificity have indicated that WRN is an aberrant structure-specific protein. The first discovered alternative DNA structure that WRN helicase can efficiently unwind in vitro is a tetraplex DNA structure (a tetrahelical DNA stabilized by guanine–guanine non-Watson–Crick hydrogen bond; also called G4 DNA, Fry and Loeb, 1999). This tetraplex is composed of $d(CGG)_n$ repeats that are expanded in the human Fragile X syndrome. G4 DNA structures may also appear at other G-rich regions such as the immunoglobulin heavy chain gene switch regions, rDNA gene clusters and telomeric repeats (Sen and Gilbert, 1988). Resolution of secondary structures by WRN suggests a role for the human RecQ helicase in removing a structural blockage ahead of the replication machinery, which otherwise would disrupt the progression of DNA replication (Barry and Alberts, 1994). Interestingly, other RecQ helicases such as human BLM and yeast Sgs1 also unwind G4 DNA (Sun et al., 1998, 1999), suggesting that this is a common feature of RecQ helicases.

WRN may have multiple roles within cells. It has been reported recently that WRN can bind to Holliday junctions and drive branch migration for a stretch of thousands of base pairs (Constantinou et al., 2000), relating WRN to the process of DNA strand exchange. Human BLM also exhibits a similar property (Karow et al., 2000b), further bridging human RecQ helicases and DNA recombination. In addition, structures such as RNA/DNA hybrids (Suzuki et al., 1997), triplex DNA (Brosh et al., 2001) and telomere repeat complexes (Ohsugi et al., 2000) can also be unwound or dissociated by WRN (Table 1).

5.2. Exonuclease

The exonuclease activity of WRN was first predicted on theoretical grounds to be located at the N-terminus having three conserved motifs similar to those of *E. coli* RNaseD and the proofreading exonuclease of *E. coli* DNA polymerase I, utilizing advanced sequence homology analysis (Moser et al., 1997; Mushegian et al., 1997). This extraordinary prediction was confirmed by the demonstration that recombinant human WRN exhibits a $3' \rightarrow 5'$ DNA exonuclease (Huang et al., 1998; Kamath-Loeb et al., 1998; Shen et al., 1998a) or controversially, a $5' \rightarrow 3'$ DNA exonuclease (Suzuki et al., 1999). As summarized in Table 2, WRN exonuclease is dsDNA dependent; its substrate requires a 3′-recessed (or 5′-overhang) DNA terminus. It does not degrade a blunt-ended DNA or single-stranded DNA. It preferentially removes a mismatched terminal nucleotide from the 3′-recessed end but is not able to degrade double terminal mismatches (Kamath-Loeb et al., 1998; Huang et al., 2000). It is also able to remove a terminal nucleotide containing 3′-PO_4, suggesting a role in repairing oxidative DNA damages (Kamath-Loeb et al., 1998). However, studies have shown that the $3' \rightarrow 5'$ WRN exonuclease is blocked by certain 3′-terminal oxidative lesions such as 8-oxo-G and 8-oxo-A (Machwe et al., 2000).

Table 1
Biochemical activities of WRN helicase

Property	Description	References
Amino acids	1432	Yu et al. (1996)
Directionality of helicase	$3' \rightarrow 5'$	Gray et al. (1997), Suzuki et al. (1997), Shen et al. (1998b)
ATPase activity	Yes	Gray et al. (1997), Suzuki et al. (1997)
DNA substrates	3′-Overhang dsDNA	Shen et al. (1998b)
	Forked DNA	Suzuki et al. (1997)
Alternate DNA substrates	Tetraplex	Fry and Loeb (1999)
	Triplex	Brosh et al. (2001)
	RNA/DNA hybrid	Suzuki et al. (1997)
	4-Way junctions	Constantinou et al. (2000)

Table 2
Biochemical activities of WRN exonuclease

Property	Description	References
Sequence homology	Proofreading exonuclease of *E. coli* DNA polymerase I	Moser et al. (1997), Mushegian et al. (1997)
	E. coli RNaseD	Moser et al. (1997), Mushegian et al. (1997)
Directionality	$3' \rightarrow 5'$	Huang et al. (1998), Shen et al. (1998a)
DNA substrates	3′-Recessed ds DNA	Huang et al. (1998), Shen et al. (1998a)
	3′-Terminally mismatched DNA	Kamath-Loeb et al. (1998), Huang et al. (2000)
	3′-PO_4 dsDNA	Kamath-Loeb et al. (1998)
	Bubble structure	Shen and Loeb (2000b)
	Extra-helical loops	Shen and Loeb (2000b)
	3- Or 4-way junctions	Shen and Loeb (2000b)
	Nicked or gapped DNA	Huang et al. (2000), Shen and Loeb (2000b)
ATP-dependence	Yes	Kamath-Loeb et al. (1998), Shen and Loeb (2000b)

Similar to the helicase activity, WRN exonuclease is also structure-specific. Since both biochemical activities reside in the same polypeptide, it is not surprising that the exonuclease activity, like the helicase, prefers alternative DNA structures such as bubble, extra-helical loop and 3- or 4-way junction DNA (Shen and Loeb, 2000b). It has been demonstrated that WRN binds tightly to these secondary structures and these structures stimulate WRN exonuclease to rapidly degrade DNA in a $3' \rightarrow 5'$ direction from the nascent blunt end or from a nascent single strand nick (Shen and Loeb, 2000b). In contrast, WRN is unable to degrade blunt-ended dsDNA and inefficiently to initiate strand degradation from a nick. These results may indicate a DNA end processing activity for WRN. It preferentially hydrolyzes alternative DNA structures such as replication forks, 3- or 4-way junctions, or at sites of DNA containing strand breakage (either single strand break or double strand break). These structures can arise spontaneously during DNA transactions, or as a result of ionizing radiation or DNA damage-induced replication fork arrest.

5.3. Oligomeric structure

Almost all discovered DNA helicases self associate as oligomers, particularly when binding to DNA substrates (Lohman and Bjornson, 1996). Determination of the oligomeric nature of a helicase provides us a mechanistic insight into DNA–helicase as well as protein(s)–helicase interactions. BLM has been shown to form a hexamer (or sometimes a tetramer) by both size-exclusion chromatography and electron microscopy (EM) (Karow et al., 1999). Evidence from size-exclusion

chromatography suggests that WRN molecules associate as trimers in the absence of DNA (Huang et al., 2000). EM pictures of WRN, or complexes of WRN and DNA, particularly the ones with alternative structures, are now urgently needed to document the trimeric structure and the interactions of WRN with alternative DNA structures.

6. WRN-interacting proteins

So far, biochemistry and genetics have failed to establish the role of WRN in cellular functions. Perhaps by identifying physiologically interacting proteins one can delineate the principal pathway(s) in which WRN participates. A series of WRN-interacting proteins have been delineated based on functional assays and/or physical interactions. Implications for possible biological roles of WRN based on these interactions are illustrated in Fig. 2. Here we discuss these proteins by order of the discovered interactions.

6.1. Replication protein A

The human replication protein A (RPA) is a ssDNA binding protein and also interacts with other proteins and modulates their activities (Wold, 1997). WRN helicase activity can be stimulated by RPA (Shen et al., 1998b; Brosh et al., 1999) and physical interactions between both proteins have also been demonstrated by co-immunoprecipitation (Brosh et al., 1999). However, further experiments are required to establish the stimulation mechanism. In addition, the use of DNA substrates with alternative structures may yield important clues to the biological function of WRN in association with RPA. In fact, RPA has been shown to be required by WRN in unwinding alternative DNA structures such as telomere repeat complexes (Ohsugi et al., 2000). At the cellular level, co-localization studies have shown that WRN foci superimpose with RPA foci in the nucleoplasm of HeLa cells treated with hydroxyurea (Constantinou et al., 2000). WRN seems to be recruited from the nucleolus to the nucleoplasm and accumulates with RPA at sites of stalled replication that is caused by depletion of cellular dNTP pool after hydroxyurea treatment.

6.2. Interaction of p53 and WRN

Direct interaction of p53 and WRN has been demonstrated by co-immunoprecipitation; the C-terminal region of WRN was considered to be important for the contact (Blander et al., 1999; Spillare et al., 1999). We have discussed previously that p53-mediated apoptosis is attenuated in WS cells (Spillare et al., 1999). Although the cellular levels of p53 in WS fibroblasts are not significantly different from that in the normal cells (Oshima et al., 1995; Spillare et al., 1999), over-expression of WRN in normal fibroblasts increases p53 levels and potentiates p53-mediated apoptosis (Blander et al., 2000). Moreover, it has been demonstrated that p53

represses the Sp1-mediated transcription of *WRN* (Yamabe et al., 1998). These findings suggest a fine-tuned feedback control between p53 and WRN, perhaps through a direct protein–protein interaction. In addition, a reduction in p53 mediated apoptosis implies that cells with damaged DNA are more likely to undergo error-prone replication and produce mutations.

6.3. PCNA and topoisomerase I

In eukaryotic cells, proliferating cell nuclear antigen (PCNA) functions in both DNA replication and DNA repair. It is a trimeric protein that acts as a scaffold protein wrapping itself around the double-stranded DNA and recruiting other proteins such as DNA polymerase δ, to sites of DNA transactions (Warbrick, 2000). A recent study found that mouse WRN co-purifies with a 17 S DNA replication protein complex through serial steps of centrifuge, chromatography and sucrose gradients, suggesting a role for WRN in replication (Lebel et al., 1999). Further characterization revealed that PCNA in the 17 S complex co-immunoprecipitates with WRN (Lebel et al., 1999) and a PCNA binding motif located within the exonuclease domain at the N-terminus of WRN is responsible for this interaction (Lebel et al., 1999). Binding between human WRN and PCNA have also been demonstrated using co-immunoprecipitation (Lebel et al., 1999; Huang et al., 2000).

DNA topoisomerase I is also a component of the 17 S replication complex. The expected protein–protein interaction between human WRN and human DNA topoisomerase I has also been demonstrated by co-immunoprecipitation (Lebel et al., 1999), suggesting a topological role for WRN when complexed with topoisomerase I. This discovery amplifies an earlier report that WS cells are sensitive to camptothecin, a DNA topoisomerase I inhibitor (Poot et al., 1999).

6.4. DNA polymerase δ

The long suspected interplay between WRN and DNA polymerases was first evidenced in a study using human WRN and yeast DNA polymerase δ (Kamath-Loeb et al., 2000). WRN enhanced the rate of nucleotide incorporation by DNA polymerase δ in primer extension assays in which PCNA is absent. The expected WRN–DNA polymerase δ interaction in humans was confirmed by yeast two-hybrid screening, indicating that the C-terminus of WRN physically interacts with the p50 subunit of human DNA polymerase δ (Szekely et al., 2000). Strikingly, native WRN co-immunoprecipitates with p50 in a cellular fraction enriched with nucleolar proteins. p125, the catalytic subunit of polymerase δ, is also present in this nucleolar fraction, suggesting a functional interaction between WRN and polymerase δ in the nucleolus. In sub-cellular localization studies of cells transfected with *WRN*, p50 and p125 redistribute to the nucleolus and co-localize with WRN, indicating that WRN perhaps recruits polymerase δ to the nucleolus (Szekely et al., 2000). Therefore, WRN may play roles in modulating the catalytic activity, as well as sub-cellular localization, of DNA polymerase δ to facilitate transactions involving DNA synthesis such as replication.

6.5. The Ku complex

The human homolog of the yeast Ku complex, Ku 86/70, is involved in many DNA biosynthetic processes including double strand break repair (Featherstone and Jackson, 1999). It has been proposed that Ku proteins bind to and stabilize DNA broken ends and recruit additional repair proteins. Using affinity chromatography, an immobilized C-terminal fragment of WRN was used to separate the Ku complex from cellular lysates (Cooper et al., 2000). In a separate study, resin-immobilized WRN proteins also selectively separated the Ku complex from HeLa cell lysates (Li and Comai, 2000). This binding was further confirmed by co-immunoprecipitation (Cooper et al., 2000; Li and Comai, 2000), and Ku 86 of the heterodimer physically contacts the N-terminal domain of WRN (Li and Comai, 2000). While the Ku complex did not affect the helicase and ATPase activities of WRN, it dramatically enhanced the $3' \rightarrow 5'$ exonuclease activity (Cooper et al., 2000; Li and Comai, 2000). The Ku complex also stimulates WRN to degrade inert DNA substrates such as ssDNA, blunt-ended or 3′-protruding dsDNA, perhaps by stabilizing the DNA terminus for the exonuclease function (Li and Comai, 2000, 2001). Based on these results, Ku might assist WRN in processing DNA ends that disrupt DNA biosynthesis. Notably, the Ku complex has recently been shown to bind tightly to G4 DNA, an alternative DNA structure that is unwound by WRN (Uliel et al., 2000).

7. The function of WRN

All lines of evidence discussed so far are compatible with the overall concept that the function of WRN in cells is to unravel alternative DNA structures and thus facilitate a variety of DNA synthetic processes. Before we draw this picture, several postulations derived from the up-to-date data should be addressed in advance, (1) WRN is an alternative DNA structure-specific enzyme; (2) WRN responds to DNA structure alterations; (3) WRN is linked to DNA synthesis (or replication); and (4) WRN deficiency causes S-phase impairment.

7.1. WRN resolves aberrant DNA structures

Aberrant DNA structures are likely to arise spontaneously at DNA segments that contain repetitive sequences such as G-rich regions at telomere, rDNA clusters and $d(CGG)_n$ repeats. These alternative structures may pre-exist in cells or are likely to arise when DNA strands become separated during replication, recombination, repair and transcription. DNA lesions such as bulky adducts and strand breaks induced by mutagens may also result in topological and structural alterations of DNA. In support of this concept are the findings that WRN cells are sensitive to the genotoxin 4-NQO (Ogburn et al., 1997) and the topoisomerase inhibitor camptothecin (Poot et al., 1992). 4-NQO is believed to form guanine adducts on DNA and might also generate DNA strand breaks in cells (Galiegue-

Zouitina et al., 1985; Yamamoto et al., 1993), while inhibition of topoisomerase activity by camptothecin results in strand breakage and altered DNA structures (Pommier et al., 1998). Biochemical evidences also indicates that WRN helicase/exonuclease resolves aberrant structures like G4 DNA (Fry and Loeb, 1999), Holliday junctions (Constantinou et al., 2000), synthetic 3- or 4-way junction DNA and extra-helical loop structure (Shen and Loeb, 2000b). In addition, DNA structure resolution-related proteins such as topoisomerase I (Lebel et al., 1999) and Ku complex (Cooper et al., 2000) have been found to interact with WRN and/or modulate WRN's catalytic activity. The fact that cells from patients with WS are not sensitive to a variety of agents that damage DNA suggests that the defect in WS is not a general defect in DNA repair. Instead, the evidences focus on the proposal that WRN is a DNA structure-resolution enzyme that assists cells to resolve tangled DNA strands and thus facilitate DNA transactions.

This hypothesis is weakened by the lack of direct in vivo evidence that WRN can resolve alternative DNA structures. While the formation of such structures are easy to observe in vitro, there are only a limited number of experiments that rigorously demonstrate their presence in eukaryotic cells. Furthermore, no missense mutation in WS patients has been reported (Moser et al., 1999) and complementation studies with specific helicase and/or exonuclease-inactivated *WRN* have yet to be demonstrated. It has been reported that introduction of human chromosome 8, where *WRN* is located, into SV40-transformed fibroblasts does not complement phenotypes of WS such as sensitivity to 4-NQO (Kodama et al., 1998). The interpretation of these negative studies may be limited by the large numbers of other mutations that presumably occur as a result of SV40-transformation. Strong support for this concept would be the demonstrated accumulation of aberrant structures in cells that lack WRN activity.

7.2. Clearing the path for DNA replication

Evidence is accumulating that DNA replication is carried out by a multiprotein complex (Waga and Stillman, 1998). Either this complex moves along the DNA or more likely, the complex is fixed to a membrane and the DNA moves through the complex. In either case, alternative DNA structures would impede replication fork movement. We suggest that a helicase(s) specialized in resolving certain types of alternate DNA structures is part of the replication machinery and functions to clear the path for the forward moving fork. Amongst these DNA helicases would be WRN for the following reasons. (1) *Xenopus* foci-forming activity 1 (FFA-1), which is required for the assembly of functional replication foci has been identified recently as a homolog of human WRN (Yan et al., 1998), suggesting a putative role of WRN in DNA replication. (2) Direct interactions between WRN and DNA polymerase δ (Kamath-Loeb et al., 2000; Szekely et al., 2000) suggest that WRN plays a role in some aspects of DNA replication. (3) WRN possesses a structure-resolving activity (Fry and Loeb, 1999; Constantinou et al., 2000; Shen and Loeb, 2000b) that could serve as a plow for the replication machinery. To illustrate this

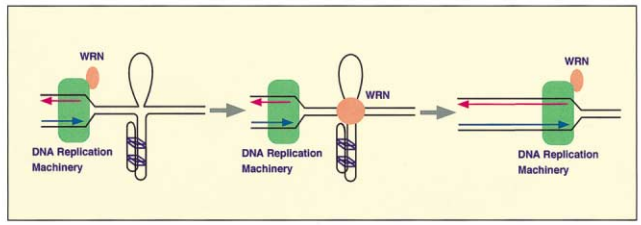

Fig. 3

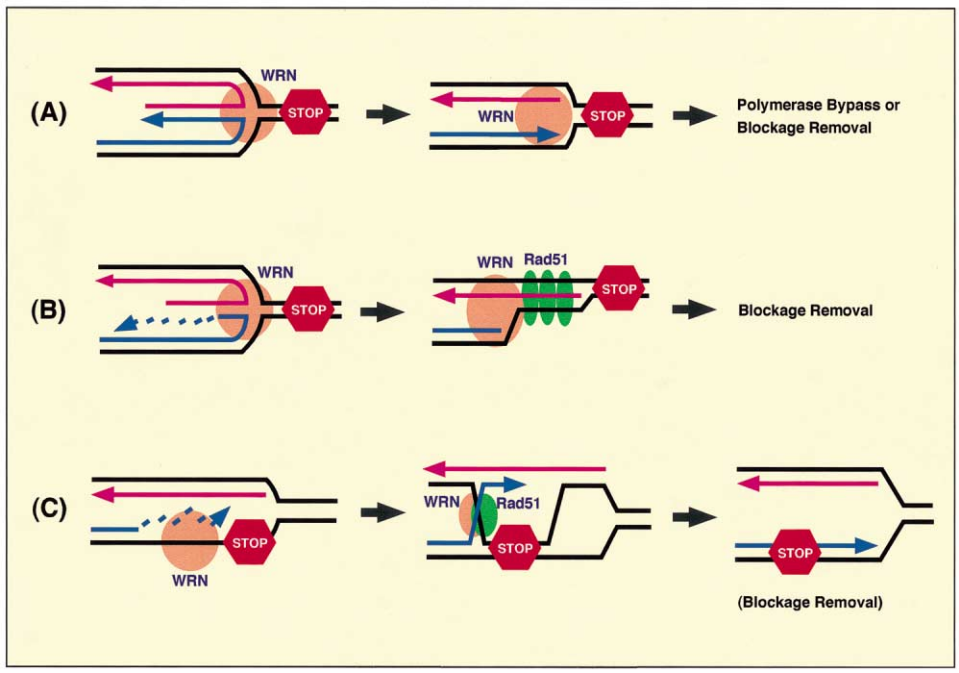

Fig. 4

Fig. 3. A model for WRN as an alternative structure resolving protein during DNA replication. An alternative structure such as G4 DNA present ahead of a progressive replication fork could impede fork movement. WRN, associated with the replication machinery (replisome), might contribute in unraveling this structural blockage to prevent the replication fork from collapse.

Fig. 4. Models for WRN in processing a stalled replication fork. (A) WRN participates in disruption of a tentative, recombinogenic 4-way junction DNA formed in a stalled replication fork. WRN dissolves the junction and helps to stabilize the fork structure for further processes such as polymerase bypass or blockage removal. (B) WRN exonuclease degrades the growing leading strand from the 3′-terminus in a stalled fork, rendering the formation of a triple-strand structure that is stabilized by tentative WRN–Rad51 complexes and facilitates further repair processes. Activity of WRN exonuclease would be dramatically increased by the formation of a 4-way junction structure. (C) The role of WRN in recombinational repair. WRN helicase/exonuclease might create a single-stranded region within a stalled fork to promote strand invasion and/or facilitate D-loop formation. Tentatively in association with Rad51, WRN might participate in branch migration, repair synthesis and/or joint molecule resolution in the recombination-mediated repair process. After restoration of the fork, the blockage could be removed by an established DNA repair process.

hypothesis, a simplified scheme showing the action of WRN on a G4 DNA that blocks the forward replication fork is presented in Fig. 3.

7.3. Processing a stalled replication fork

The progression of DNA replication forks can be arrested frequently due to structural blockage or blocking DNA lesions that need to be excised. This blockage could be a signal for intervention by a bypass polymerase (Goodman and Tippin, 2000). In the absence of bypass, collapse of the replication machinery results in four strands of DNA tangling together and leading to aberrant structures such as 3- or 4-way junction. These structures may further induce aberrant recombinations that give rise to genetic alterations if not resolved appropriately. Lessons from bacteria (see earlier discussions) indicate that RecQ helicase may function in processing and/or restoring disrupted replication forks. The model in Fig. 4A is based on a general assumption that an abandoned DNA replication fork increases the chance of forming a 4-way junction DNA that may eventually lead to illegitimate strand exchange. WRN disrupts Holliday junctions and thus prevents unwanted strand exchange. As a consequence, WRN helps to stabilize the fork structure and increase the time available for DNA repair.

Results in bacteria indicate that RecQ together with RecJ displaces and degrades the lagging strand at an UV-induced stalled replication fork (Courcelle and Hanawalt, 1999). The recessed fork presumably will be stabilized in a triple-stranded form by RecA filaments prior to damage repair. WRN possesses a unique $3' \rightarrow 5'$ exonuclease activity and this activity has been characterized as structure-specific, particularly at the 4-way junction DNA (Shen and Loeb, 2000b). It is likely that the formation of a 4-way junction structure within a stalled replication fork is prevented by degradation of the leading strand by the $3' \rightarrow 5'$ WRN exonuclease (Fig. 4B). A tentative WRN–Rad51 (a human RecA homologue) complex might stabilize the resulting triple-stranded structure in the fork after WRN processing, prior to removal of the DNA damage or structural blockage (Fig. 4B).

Recombinational repair at a stalled replication fork is an attractive postulation, although the exact role of RecQ in this process is still elusive even in *E. coli* (Cox, 1998). Taking into account the capability of *E. coli* RecQ to initiate and disrupt homologous recombination (Harmon and Kowalczykowski, 1998) and the ability of WRN to affect Holliday junctions (Constantinou et al., 2000), we propose a model in which WRN functions in recombination-mediated gap repair after replication fork stalled (Fig. 4C). WRN is able to initiate helicase unwinding and exonuclease degradation from a small gap or a nick (Huang et al., 2000; Shen and Loeb, 2000b) or within a synthetic replication fork (Shen and Loeb, unpublished results). The WRN helicase/exonuclease might create a single strand segment in the stalled fork to facilitate subsequent strand invasion. At the second stage, WRN together with Rad51 and RPA might promote the formation of a D-loop and joint molecule. WRN might also assist Rad51 in directing the branch migration and could facilitate recombinational DNA synthesis to fill in the gap. Finally, following joint molecule

resolution, potentially by WRN, the replication fork would be restored and the DNA lesion would be ready for further repair (Fig. 4C).

8. Yeast as a RecQ model

Unlike higher eukaryotes that have multiple RecQ helicases, yeast such as *S. cerevisiae* or *S. pombe* contains only one RecQ homolog, i.e. *sgs1* (Gangloff et al., 1994; Watt et al., 1995) or *rqh*1 (Stewart et al., 1997), respectively. Similar to human RecQ-deficient syndromes, yeast cells lacking RecQ exhibit genetic instability. The *sgs*1 mutants show phenotypes such as hyper-recombination, increased chromosome missegregation and a shortened life span (Watt et al., 1996; Sinclair et al., 1997), and the *rqh*1 mutants are hypersensitive to DNA damage (Stewart et al., 1997). Notably, these yeast homologues lack a $3' \rightarrow 5'$ exonuclease, which may be a key in deciphering the age-specific associations of WRN.

The *sgs*1 mutant was first discovered as a suppressor of the slow-growth phenotype of *top*3 mutants that are deficient in DNA topoisomerase III (Gangloff et al., 1994). Interestingly, *top*3 mutants also exhibit genomic instability, manifested as hyper-recombination between repetitive DNA elements; this phenotype can be suppressed by the *sgs*1 allele. Given that Sgs1 binds to Top3, both proteins might function in concert to resolve topologically altered DNA structures that result from the functional play of Sgs1. Sgs1 also interacts with Top2 and both of them might again act in concert to maintain faithful chromosome segregation (Watt et al., 1995). The RecQ–topoisomerase link observed in yeast is reminiscent of the WRN–TopI interaction for which the biological role is still unknown. Nonetheless, the hyper-recombination phenotype of *sgs*1 mutants has been utilized for studying human RecQ in a complementation assay, showing that both WRN and BLM are able to restore the mutant phenotypes (Yamagata et al., 1998). Most recently, Sgs1 has been shown to be epistatic to DNA mismatch repair (MMR) in suppression of gross chromosomal rearrangement including translocations and deletions (Myung et al., 2001). Such a functional redundancy between Sgs1 and MMR also appears in reducing 'homeologous' recombination that occurs between DNA sequences with about 90% homology. Similar to *E. coli* RecQ, Sgs1 also functions as a suppressor of illegitimate recombination in *S. cerevisiae*.

An interesting aging-related phenotype displayed in yeast that is also accelerated in *sgs*1 mutants is the accumulation of extrachromosomal rDNA circles (ERCs) (Sinclair and Guarente, 1997). rDNA repeats are G-rich and may form secondary structure such as G4 DNA. The putative G4 structure might interfere with DNA replication or transcription and render DNA to be recombinogenic. Aberrant recombination might occur as a result to form extrachromosomal DNA circles. Sgs1 may be involved in processes that resolve secondary structures or suppress aberrant recombinations. However, there is no evidence for a similar phenomenon in human cells during aging in culture. Yeast lacks the Alu sequence and a wide variety of repetitive sequences present in human cells. If an increase in the

formation of extrachromosomal circular DNA occurred at all repetitive sequences in human cells and was not limited to rDNA, it would be difficult to detect.

Yeast has provided a well-characterized model to study the postulated checkpoint functions of RecQ. It has been reported recently that Sgs1 participates in a checkpoint that responds to stalled replication forks in S-phase (Frei and Gasser, 2000). Similarly, WRN might play the same role as an S-phase checkpoint protein since WRN is likely to process stalled replication forks. WRN might also have a G1- or G2-phase checkpoint function, as WRN may serve as a recruiter of p53 to sites of DNA lesions.

9. The mouse model

If only one can mimic the WS phenotype in mice, one may obtain key information of the molecular basis of WRN and perhaps the aging phenotype. The mouse *Wrn* gene on chromosome 8A4 is syntenic to the human *WRN* gene on chromosome 8p (Goto et al., 1992; Imamura et al., 1997) and its cDNA sequence shares greater than 70% homology with the human gene (Imamura et al., 1997). The recombinant gene product of mouse *Wrn* isolated from insect cells using the baculovirus expression system exhibits identical biochemical activities to its human counterpart, including the $3' \rightarrow 5'$ helicase as well as $3' \rightarrow 5'$ exonuclease activities (Huang et al., 2000). Both genetic and biochemical similarities of mouse and human WRN suggest that mice could serve as an important model for studying human WS.

The first *Wrn* knockout mouse was created by deletion of a portion of the helicase domain (Lebel and Leder, 1998). This deletion apparently abolishes WRN helicase activity but the exonuclease domain is retained and could still be active. In this genetic background, it would be interesting to examine the exonuclease activity of the mutant WRN and determine phenotypic, as well as genetic changes in cells harboring the helicase-deficient alleles. The WS mice appear normal during their first year of life (Lebel and Leder, 1998). However, embryonic stem cells of homozygous WS mice exhibit increased sensitivity to certain genotoxins such as 4-NQO and camptothecin when compared with wild-type cells. In addition, embryonic fbroblasts derived from homozygous WS mice show premature loss of proliferative capacity. It seems that deletion of WRN helicase activity alone results in mouse cells with some of the cellular phenotypes of WS. However, we lack evidence of an aging phenotype in these mice.

A second *Wrn* knockout mouse created by deletion of the C-terminus of WRN protein including a portion of the helicase domain has been reported recently (Lombard et al., 2000). Unlike the first WS mouse, embryonic fibroblasts from the homozygous mutants did not exhibit increased sensitivity to 4-NQO and camptothecin. The proliferative capacity was also examined in splenocyte cultures derived from the mutant animals and appeared to be normal as compared with the wild-type cells. These mice are capable of living beyond 2 years of age. However, the homozygous WRN mutations accelerate mice mortality in a p53-null back-

ground, perhaps indicating that p53 and WRN play synergistic roles in determination of life span (Lombard et al., 2000).

In addition to these knockout mice, one line of transgenic mice expressing human mutant WRN harboring a missense mutation (K577M) has been created recently (Wang et al., 2000). The K577M mutation in WRN inactivates the ATPase and helicase activities, but not the exonuclease activity (Gray et al., 1997; Huang et al., 1998; Shen et al., 1998a). A similar substitution in *E. coli* UvrD helicase, interestingly, results in a dominant-negative mutation (George et al., 1994). Such an effect is also present in the transgenic mice. Tail-derived fibroblasts from the human K577M WRN mice exhibit characteristic WS phenotypes such as increased sensitivity to 4-NQO and reduced replicative life span. While the results suggest that the helicase activity is essential to the function of WRN with respect to DNA biosynthesis, they do not shed light on the role of the exonuclease. For example, do mutations in the helicase abolish the association with other proteins that are necessary for exonucleolytic activity?

The mouse model could be very helpful in addressing many questions ranging from the molecular essentiality of WRN catalytic activities to the pathogenesis of WRN mutation-induced premature aging that we could not easily accomplish by studying human cell cultures. However, species-specific difference of WRN properties should also be considered carefully before we draw any conclusion. For example, mouse WRN distributes all over the nucleoplasm (Marciniak et al., 1998; Wang et al., 2000), whereas the human counterpart predominantly concentrates in the nucleolus (Gray et al., 1998; Marciniak et al., 1998). Interestingly, human WRN expressed in mice is also distributive in the entire nucleus, losing its characteristic of nucleolar localization (Wang et al., 2000). To date, WRN null mice still lack typical human WS phenotypes. Nonetheless, genetic and/or molecular studies of mice carrying multiple genetic backgrounds such as the established WRN/p53 null mice or the postulated WRN/hTERT mice could serve to address specific functions of WRN that may be associated with aging.

10. Summary

Despite the recent exciting advances in the genetics, biochemistry and cell biology of WRN, we still lack a thorough understanding of its function in normal cells. We have been hampered by the lack of missense mutations, the multiple catalytic activities of WRN and the redundancies of helicases that might substitute for WRN. Never the less, the evidence so far accumulated indicates that WRN functions in the resolution of alternative DNA structures that form as a result of DNA damage or during normal DNA replication. Mutations in WRN prevent the resolution of these structures, hamper DNA synthetic process and result in genetic instability. We present several models to account for the function of WRN in resolving alternative DNA structures and the consequences of deficits in these functions. We offer these models in the hope that they will stimulate experiments to test their validity and to formulate new models. While the proposed models account

for some of the cellular phenotypes exhibited by mutations that abolish WRN, they are totally inadequate for explaining the cardinal phenotype of WRN, namely, the early onset of age-related pathologies.

Acknowledgements

We are grateful to Dr Ashwini Kamath-Loeb for her critical reading of this manuscript. Research reported in this article from the author's laboratory was supported by grant CA-77852 from the National Institute of Health.

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