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Review

Current advances in unraveling the function of the Werner syndrome protein

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Abstract

Werner syndrome (WS) is an autosomal recessive premature aging disease manifested by the mimicry of age-related phenotypes such as atherosclerosis, arteriosclerosis, cataracts, osteoporosis, soft tissue calcification, premature thinning, graying, and loss of hair, as well as a high incidence of some types of cancers. The gene product defective in WS, WRN, is a member of the RecQ family of DNA helicases that are widely distributed in nature and believed to play central roles in genomic stability of organisms ranging from prokaryotes to mammals. Interestingly, WRN is a bifunctional protein that is exceptional among RecQ helicases in that it also harbors an exonuclease activity. Furthermore, it preferentially operates on aberrant DNA structures believed to exist in vivo as intermediates in specific DNA transactions such as replication (forked DNA), recombination (Holliday junction, triplex and tetraplex DNA), and repair (partial duplex with single stranded bubble). In addition, WRN has been shown to physically and functionally interact with a variety of DNA-processing proteins, including those that are involved in resolving alternative DNA structures, repair DNA damage, and provide checkpoints for genomic stability. Despite significant research activity and considerable progress in understanding the biochemical and molecular genetic function of WRN, the in vivo molecular pathway(s) of WRN remain elusive. The following review focuses on the recent advances in the biochemistry of WRN and considers the putative in vivo functions of WRN in light of its many protein partners.
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Keywords: Werner syndrome; Genomic instability; DNA replication; DNA repair; Recombination; Protein partners

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1. Introduction

Phil Hanawalt has championed the use of bacterial genetics to define functions of disease associated human genes. This approach has contributed much to his successes in analyzing mechanisms of DNA repair, the field that he has fathered from its infancy. His most recent efforts have focused on UV-induced DNA damage and he has established the concept of strand specificity in DNA repair. Hovering in the background have been his studies on DNA helicases, the enzymes that separate the DNA strands and facilitate DNA polymerization. Work on RecQ helicases started when Hiroaki Nakayama and Phil Hanawalt identified the prototype RecQ helicase, *Escherichia coli* RecQ. Phil is so fond of telling how Hiroaki Nakayama named the enzyme. At that time a series of recombination enzymes were being identified, each adding a new letter, RecA, B, C, ..., P. RecP might have been next, except Nakayama lived in Japan and his home city was Kyushu, which they transcribed to Q. It is ironic that Phil now presents evidence that many of these bacterial recombination enzymes function in other DNA processes, and RecQ is not an exception. Studies on RecQ helicases could be an arcane field except for an important consideration: inherited mutations in these enzymes are causally associated with human diseases. There are five human homologs of *E. coli* RecQ, RECQ1, RECQ2/BLM, RECQ3/WRN, RECQ4 and RECQ5. Mutations in three of these, BLM, WRN, and RECQ4, result in genetic instability syndromes, Bloom's syndrome (BS), Werner's syndrome (WS), and Rothmund–Thomson syndrome (RTS), respectively, and are manifested by tumor predisposition and/or premature aging. Inherited mutations in the Werner helicase are associated with the premature onset of a number of age-related problems and an increased incidence of specific human tumors. Mutations in BLM helicase are linked with elevations of sister chromatid exchanges and a substantial increase in a wide spectrum of malignancies. Mutations in RECQ4 helicases give rise to Rothmund–Thomson syndrome, a genetic instability syndrome characterized by skin and skeletal abnormalities and an above average incidence of cancer. In this article, dedicated to Phil Hanawalt, we will review the molecular studies on Werner syndrome, a fascinating disease that may offer clues to human aging and to lineage specificity in human cancers. We know much about the biochemistry of the WS protein, yet we are unable to delineate the role of this enzyme in cellular processes. Perhaps Phil's admonition may be correct: we may be able to understand the role of WRN in DNA transactions only after we understand the function of RecQ in *E. coli.* Studies on the RecQ family of proteins in genetically tractable organisms may give

us insights into major human problems of aging and cancer.

The corresponding author is not a product of Phil Hanawalt's laboratory, he is an observant of how a laboratory should be run, one designed to both create knowledge and to mentor the careers of future scientists. Phil is exceptional in these endeavors: he has a vision of the importance of DNA repair in the cells armamentarium against endogenous and environmental DNA damage, and he has been unwavering in supporting the careers of colleagues. His generosity to the scientific community is documented and embedded in the many conferences he has organized, culminating in the International Conference on Environmental Mutagenesis. Phil traditionally ends his lectures with pictures from his laboratory retreat including current members, alumni, and visiting scientists that are selected to "keep them honest." I was one of the visitors to his retreat and immediately recognized their importance; we mimicked his wonderful tradition and invited Phil to be one of our early critics.

2. Werner syndrome

The Werner syndrome (WS) protein, WRN, is a member of the RecQ family of DNA helicases [1] that are widely distributed in nature and believed to play central roles in maintaining the genomic stability of organisms ranging from prokaryotes to mammals [2]. *WRN* encodes a single polypeptide of 162 kDa that contains 1432 amino acids. Individuals harboring mutations in *WRN* have a rare, autosomal recessive genetic disorder manifested by an early onset of symptoms characteristic of aged individuals. Generally, Werner syndrome becomes apparent by the failure of the growth spurt at adolescence followed by an accelerated development of atherosclerosis, arteriosclerosis, cataracts, osteoporosis, soft tissue calcification, premature thinning, graying, and loss of hair. In addition, WS patients display a higher incidence of 'late onset' (Type II) form of diabetes mellitus, as well as an elevated cancer frequency that is largely restricted to those of mesenchymal origin. The age of death varies between approximately 30 and 65 years, with a mean of 47 years, and usually results from cancer or cardiovascular disease [3–6]. Since WS patients display such a remarkable number of progeroid phenotypes (with the exception of the lack of degeneration of the central nervous system and a reduction in immune function), WS provides a unique model for the studying normal aging as well as age-associated diseases in that it might promote new mechanistic insights that are experimentally tractable.

Fibroblast cultures from WS patients display a prolonged S-phase [7], attenuated replicative potential [8,9] that correlates with a faster rate of decline in the mitotic fraction per population doubling [10], as well as a variety of chromosomal abnormalities including reciprocal translocations, deletions and inversions [11]. Furthermore, in addition to reports on increased levels of homologous recombination [12], some WS cell lines also show aberrant mitotic recombination [13]. There is an elevated level of large spontaneous deletion mutations (>20 kb) coupled with sensitivity to a range of DNA damaging agents [14]. Thus, WS can be classified as a genomic instability syndrome and *WRN* might be classified as a lineage specific tumor suppressor gene.

3. WRN protein

Interestingly, such a diverse collection of cellular and organismal phenotypes of WS is caused by the loss-of-function mutations in a single gene product located at chromosomal position 8p12 [1,15]. WRN is a DNA-dependent ATPase that uses the energy from ATP hydrolysis to unwind double-stranded DNA in the 3′–5′ direction with respect to the single strand that it binds [16–18]. However, unlike other known members of the human RecQ family, WRN contains three conserved exonuclease motifs with significant sequence similarity to the 3′–5′ proofreading domain of *E. coli* DNA polymerase I as well as RNaseD [19], and thus is the only known member of this family to possess a 3′–5′ exonuclease activity [20,21]. In addition to the N-terminal exonuclease domain that spans amino acids 78 through 219, other major domains of WRN are the centrally located RecQ helicase domain covering amino acid residues 569 through 859 and consisting of seven conserved motifs, a direct repeat of 27 amino acids between the exonuclease and helicase domains, a putative transcription activation domain (amino acids 315 through 403), and the C-terminal nuclear localization element (amino acids 1370 through 1375) (reviewed in [22]). The C-terminal region of WRN also accommodates the conserved RQC domain (*RecQ c*onserved)

that includes the nuclear localization signal-dependent nucleolar targeting sequence, as well as the HRDC (*H*elicase and *R*nase*D* *C*-terminal) domain believed to play a role in DNA binding [23,24]. As in other members of the superfamily 1 and 2 helicases, motifs I and II (Walker A and B motifs, respectively) of the WRN helicase domain contain the amino acids critical for interacting with MgATP/MgADP [22,25,26]. Analysis of more than 30 *WRN* mutations identified in WS patients thus far indicates that all of the mutations give rise to truncated WRN proteins with a loss up to 1256 amino acid residues that invariably includes the C-terminal nuclear localization signal [27]; these mutations (nonsense, frameshift, or insertion/deletion) inactivate both copies of the WS gene and lead to the loss of detectable protein [28]. The phenotypes of cell lines from heterozygous carriers of the mutated WRN gene with reduced levels of both WRN protein and helicase activity suggests that a WRN dosage effect may modulate WS pathogenesis [29]; however, so far no pathology has been established in heterozygous carriers.

4. Biochemical properties of WRN protein

WRN protein is unique among the five human RecQ members in that it is a bipartite and bifunctional enzyme: not only is it an ATP-dependent 3′–5′ helicase and a DNA-dependent ATPase characteristic of all RecQ family helicases, but unlike any other member of the RecQ family, it possesses a 3′–5′ exonuclease activity [16–21]. The two functions of the enzyme are functionally and physically separable from each other. Amino acid substitutions that inactivate the exonuclease activity of WRN do not interfere with its helicase function, while mutant proteins with amino acids substitutions or deletions in the helicase/ATPase domain are still able to digest DNA exonucleolytically but fail to unwind it [20,21]. Similarly, recombinant N-terminal fragments display exonuclease but no helicase activity, while C-terminal fragments that lack the exonuclease domain retain the helicase function [30–32]. However, stimulation of the exonuclease activity by ATP hydrolysis [33] suggests some cooperativity between the ATPase and exonuclease functions of WRN, and underlines the proposal that full function and regulation of catalytic activity may require the presence of other regions of the protein in addition to these minimal domains [34].

The ATPase activity of WRN is DNA-dependent [16] and is significantly stimulated by long stretches of ssDNA (>250 nt), although short ssDNA oligonucleotides and dsDNA can also act as stimulators of ATP hydrolysis [35]. The correlation between the maximum k_{cat} value of 200 min^{-1} for ATP hydrolysis and the ability of WRN to translocate along long stretches of ssDNA without additional binding steps suggests processive translocation of WRN protein along ssDNA [35]; however, as discussed below, this processivity does not apply to the helicase and exonuclease activities of the protein.

The ATP-hydrolysis driven 3′–5′ helicase activity of WRN [16–18] shows relatively poor processivity on long DNA duplexes [36] and is able to unwind only short DNA duplexes (≤53 bp) in the absence of auxiliary co-factors [16,17]. The initial rate of the unwinding reaction displays a hyperbolic dependence on ATP and Mg^{2+} concentrations suggesting that WRN helicase activity is not cooperative with respect to ATP concentration [37]. Similar to many phosphotransferases, Mn^{2+} or Ni^{2+} can substitute for Mg^{2+} as a co-factor, whereas both Fe^{2+} and Cu^{2+} profoundly inhibit the helicase activity in the presence of Mg^{2+} [37].

A characteristic feature of WRN helicase is its specificity in unwinding diverse DNA substrates, some of which deviate from the canonical B-form duplex DNA that could potentially interfere with cellular processes such as replication or transcription, thus giving rise to genomic instability. A physiologically important alternative DNA structure that WRN can efficiently unwind in vitro is quadruplex DNA (also called G4 tetraplex DNA) [38], which is held together by guanine–guanine Hoogsteen base pairing and stabilized by monovalent alkali cations [39]. Such G-rich DNA sequences that readily form quadruplex structures under appropriate in vitro conditions are widely distributed throughout the genome and are found, among other places, at immunoglobulin switch regions and rDNA gene clusters [39], as well as at telomeric repeats [40]. It is possible that these structures might also form in vivo and have specific functions in regulation of gene expression or genetic stability. In addition, WRN can resolve triplex DNAs [41] that are most readily formed on polypurine:polypyrimidine sequences and have been demonstrated both in chromosomes

[42] and nuclei [43], as well as forked DNA molecules [17], partial DNA duplexes with a single-stranded 3′-overhang [20], D-loops [44], and partial DNA–DNA and DNA–RNA duplexes [17]. Furthermore, it has been demonstrated that WRN is capable of branch-migrating Holliday junctions over several kilobases [45], a remarkable feat considering that WRN normally displays poor processivity [36]. Taken together, these substrate requirements suggest that a major function of WRN is to alleviate blocks during DNA synthetic processes.

Biochemical data on the exonuclease activity of WRN, which resides at the N-terminus, indicate a $3' \rightarrow 5'$ directionality for exonucleolytic DNA degradation and low processivity [20,21,33]. In contrast to what has been observed with WRN helicase, Zn^{2+} can substitute for Mg^{2+} as a co-factor for WRN exonuclease in the absence of ATP, and could act as a molecular switch, converting WRN from helicase to exonuclease in vitro [37]. The presence of a Zn^{2+} binding domain is suggested by the Zn^{2+}-dependent stimulation of exonuclease activity in N-terminal WRN fragments and further supported by the structure of the sequence-related DNA polymerase I $3' \rightarrow 5'$ exonuclease domain which is proposed to function by a two-metal ion mechanism [46]. Thus, it is conceivable that the catalytic activities WRN are regulated and its cellular functions modulated by metal ion availability [37]. Early studies on simple substrates showed that WRN exonuclease degrades double-stranded DNA or DNA–RNA heteroduplexes containing 3′-recessed ends more efficiently than double-stranded duplexes with blunt ends, partial duplexes with 5′-recessed ends, or ssDNA for which it has essentially no activity [47]. Interestingly, the introduction of certain defined structures such as a centrally located bubble or an extra-helical loop allows the initiation of digestion from blunt ends [30,47]. Moreover, as is the case with its helicase activity, the preferred "activators" for the WRN exonuclease are unusual DNA structures: bubble-containing duplex DNA, DNA with single-stranded loop, stem-loop DNA molecules, as well as three-way and four-way DNA junctions [47]. Since both the helicase and the exonuclease activities of the WRN protein reside on the same polypeptide [20], this preference for alternative DNA structures is not surprising. The fact that a single mismatched terminal nucleotide from a 3′-recessed end is a more effective substrate for the exonuclease

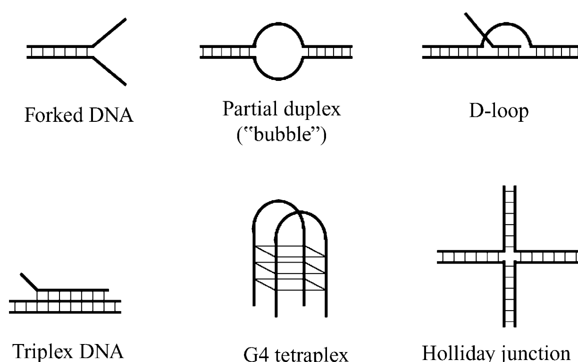

Fig. 1. Some alternate DNA structures as substrates for WRN. WRN shows substrate specificity towards alternate DNA structures thought to exist in vivo as intermediates in specific DNA transactions such as replication (forked DNA), recombination (Holliday junction, triplex and tetraplex DNA), and repair (partial duplex with single stranded bubble).

activity of WRN than is an otherwise identical non-mismatched molecule [21,33] suggests a role in 'proofreading' akin to the proofreading activities of certain DNA polymerases. Furthermore, WRN exonuclease is active at nicks and gaps [21] and on certain modified bases such as uracil and hypoxanthine [32]. Fig. 1 schematically depicts some of the substrates of WRN protein.

The function of the WRN exonuclease has not been established. Since WRN is able to remove a terminal nucleotide containing $3'\text{-}PO_4$, it is suggested that it may play a role in repairing oxidative DNA damage [33]; however, certain 3′-terminal oxidative modifications and bulky lesions in DNA block the exonuclease activity [32]. On the other hand, association with Ku enables WRN exonuclease to excise different blocking lesions [48]. Although the exonuclease activity of WRN can be observed in the absence of ATP [33], it is nevertheless dramatically stimulated on every substrate tested by ATP hydrolysis [30,33], suggesting cooperativity between the ATPase and exonuclease domains. On the other hand, mutant WRN proteins lacking the entire ATPase/helicase domain still retain exonuclease activity [32], suggesting a functional independence of helicase and exonuclease activities.

An important question that remains unanswered is whether or not the helicase and exonuclease activities of WRN function coordinately in a common molecular pathway. While similar binding affinities and substrate

preferences suggest coordinate action, it is also possible that the separate activities of the protein may sequentially play independent roles in discrete steps of a single pathway. Alternatively, though less likely, the helicase and exonuclease activities may operate separately in two distinct DNA metabolic pathways. While distinct structure-specific DNA binding domains [49] and separable helicase and exonuclease activities of mutant WRN proteins [20,21,30,31,33] support a distinct pathway hypothesis, recent reports of an enhanced exonuclease function in the concerted DNA binding and exonuclease activities on partially melted duplex DNA [30] and the simultaneous action of WRN helicase and exonuclease on opposite ends of a long forked DNA duplex as well as their cooperation in the subsequent separation of the strands [50] favor the coordinated action model. In addition, it has been reported that a similar cooperation exists in the removal of the invading strand of a long D-loop [44,51], which is an early intermediate in recombination pathways [52]. Furthermore, there is evidence indicating that, in addition to a structural role it plays independent of its enzymatic activities, balanced helicase and exonuclease activities of WRN are required in DNA repair via homologous recombination [53].

Although these studies suggest that WRN helicase and exonuclease indeed act in concert to process alternative DNA structures, how this coordination is achieved remains a puzzling question. Earlier studies [18,21,33] have shown that the domains have opposing specificities: whereas WRN helicase function requires a single-stranded region $3'$ to the duplex to be unwound and proceeds in a $3' \rightarrow 5'$ direction as defined by the single-stranded template it binds, the exonuclease function requires a single-stranded region $5'$ to the duplex to be degraded while it advances with a $3' \rightarrow 5'$ polarity as defined by the strand on which it acts. Depending on the substrate upon which WRN acts, the two activities appear to move towards or away from each other [22,30,50]. It has been hypothesized that WRN might warp or twist the DNA substrate to allow the helicase and exonuclease domains to face the opposite ends of the DNA and thus proceed in the same direction [22], while a similar scenario envisions a looping or bending mechanism which can bring a distant DNA end or nick in close proximity to the static WRN protein [30]. Another possibility is that WRN, acting as an oligomer, is able to span DNA stretches long enough to be able to simultaneously process opposite ends of the same DNA molecule [50]. Alternatively, WRN helicase, upon binding to a partially melted region of duplex DNA, may facilitate the movement of the bubble either toward or away from the end or nick slated for exonucleolytic degradation [30]. These multiple conjectures emphasize the importance of establishing the three dimensional structure of WRN in complex with different DNA substrates.

Further complicating the matter is the fact that the quarternary structure of WRN is controversial. The initial rate of unwinding increases with WRN concentration, suggesting the formation of a functional multimeric enzyme complex, while pre-steady state conditions reveal an initial burst phase amplitude at a 1:1 ratio between WRN and the DNA substrate, suggesting an active monomeric form of the helicase [37]. Conversely, full-length WRN was proposed to form a trimer based on observations with size-exclusion chromatography [54], whereas another study utilizing gel-filtration chromatography and atomic-force microscopy showed that a minimal exonuclease domain of WRN existed in a trimer-hexamer equilibrium in the absence of DNA with the trimer form being stabilized in the presence of DNA or PCNA [55]. However, WRN and other RecQ helicases have been postulated to exhibit a dynamic change in subunit structure that affects their activity and function (reviewed in [51]).

5. WRN and its protein partners

Consistent with the bipartite and bifunctional nature of WRN and its numerous substrates that resemble various intermediates in DNA replication, recombination, and repair processes, WRN has been shown to physically and functionally interact with a variety of proteins. Since genetics and biochemistry have so far failed to establish a definitive role of WRN in cellular function, a thorough examination of physiologically important WRN interactors may help delineate the principal pathway(s) in which WRN participates. However, the large number of WRN-interacting proteins and the fact that many of these interactors participate in a variety of DNA synthetic pathways have so far prevented a clear definition of the cellular function of WRN based on associations (Fig. 2).

WRN

Replication

S-phase
Replication foci
DNA substrates
(forks, bubbles)
PCNA
Topo-1
DNA Polδ
RPA
FEN-1

Recombination &
Telomere maintenance

DNA substrates
(Holliday junctions,
Tetraplexes, forks,
D-loops)
Rad51/52
Mre11
TRF1/2

Repair

DNA substrates
(partial duplex,
bubble)
p53
Ku/DNA-PK
DNA Polβ/APE-1
PARP-1

Fig. 2. Evidence for the proposed in vivo functions of WRN. All lines of evidence discussed so far are compatible with the overall role of WRN in resolving alternative DNA structures, facilitating a variety of DNA synthetic processes. However, the large number of WRN-interacting proteins and the fact that many of these interactors participate in a variety of DNA synthetic pathways prevent the formulation of the precise cellular function of WRN.

5.1. Replication proteins

5.1.1. PCNA and topoisomerase I

One of the earliest demonstrations of physical and functional interaction of the WRN protein with members of the replication machinery came from co-immunoprecipitation studies, which identified WRN as part of the 17S multiprotein DNA replication complex, and established PCNA and topoisomerase I as the two WRN-interacting components [56].

PCNA is a trimeric scaffolding protein akin to the *E. coli* β-clamp, and is involved in both DNA replication and repair processes. It wraps itself around the DNA duplex and recruits other proteins, including DNA polymerase δ, to form so called replication factories (reviewed in [57]). The exonuclease domain of the WRN protein contains a region that is homologous to the PCNA-binding motif [56,58] found in many proteins involved in DNA replication and repair, such as FEN-1 and DNA ligase 1 [57,59]. Recently, it has been demonstrated that WRN and PCNA co-localize at replication foci, suggesting a physiological interaction between them in cycling primary cells [58]. Since this interaction occurs through the conserved PCNA-binding motif, it is proposed that a competitive interaction between PCNA-binding replication factors and WRN may play an important role in regulating the activity of WRN [58]. However, its interaction with the PCNA does not assign a definitive role to WRN in cellular processes because PCNA is involved in both DNA replication and repair. Moreover, the size of these foci could accommodate thousands of protein molecules and thus co-localization does not imply a direct molecular association.

Topoisomerases change the linking number of DNA during DNA replication to relieve the torsional stress caused by the advancing replication fork. The WRN-topoisomerase I association implies a topological role of WRN that might explain the sensitivity of WS cells to a potent DNA topoisomerase I inhibitor, camptothecin, during G_2 and S phases of the cell cycle [60,61]. Recent evidence indicates that WRN physically interacts with topoisomerase I through two regions located at the C- and N-termini of the WRN polypeptide and stimulates the ability of topoisomerase I to relax negatively supercoiled DNA, while in a reciprocal functional interaction topoisomerase I inhibits the ATPase activity of WRN [62]. WS cells also show hypersensitivity to chromosome damage induced by topoisomerase II inhibitors during the G_2 phase of the cell cycle, suggesting defective decatenation checkpoint that could contribute to genomic instability through imperfect segregation of sister chromatids and subsequent chromosome breakage in the absence of WRN [63].

5.1.2. DNA polymerase δ

The association of WRN with the major replicative DNA Polymerase δ (Polδ) more directly suggests the involvement of WRN in DNA replication. The addition of increasing amounts of WRN dramatically stimulates the rate of nucleotide incorporation by yeast DNA Polδ in primer extension assays in which PCNA is absent [64]. Using yeast DNA Polδ, it was demonstrated that the enhancement in primer extension is dependent on the presence of the Pol32 subunit. The finding that WRN does not stimulate primer-extension by the Polδ–PCNA complex, which is required for efficient replication in vivo, argues that WRN is not involved in normal processive DNA synthesis [64]. The addition of the WRN helicase allows Polδ to traverse hairpin and G-quadruplex structures that normally impede the translocation of replication complexes, and allows the synthesis of full-length DNA [65]. A role for WRN in resolving alternative DNA structures is reinforced by studies of Courcelle and Hanawalt showing that in *E. coli*, RecQ is required to process DNA at blocked replication forks [66]. The resolution of these

secondary structures is of biological importance for they can induce polymerase stalling and prolong the S-phase as observed in WS cells lacking WRN protein [67].

Studies using yeast two-hybrid screening indicate that the C-terminal region of WRN physically interacts with the p50 subunit of the human DNA polymerase δ, and co-immunoprecipitates with p50 and p125 subunits [68]. Furthermore, ectopically introduced tagged WRN co-localizes with p50 and p125 in the nucleolus of HeLa cells, indicating a role for WRN in sub-cellular localization in addition to its modulation of catalytic activity [68]. This dichotomy between stimulation and binding of WRN to Polδ may indicate that WRN binds to one subunit and stimulation is dependent on the presence of the other subunit. All in all, because Polδ participates in both DNA replication and DNA repair, its association with WRN does not reveal the specific pathway in which WRN partakes but merely hints at its role as a "genomic caretaker."

5.1.3. RPA

Another important protein that associates with WRN and forms a functional complex is the replication protein A (RPA). Human RPA is a heterotrimeric, single-stranded DNA binding protein required for DNA replication, recombination, and repair (reviewed in [69]). Direct physical interaction between WRN and RPA, demonstrated by their co-immunoprecipitation [36] and through enzyme-linked immunosorbent assay (ELISA) [70], markedly stimulates the DNA helicase activity of the WRN protein [36,70] and increases its ability to unwind forked telomeric DNA structures [50,71]. While WRN alone cannot unwind partial duplexes longer than 40 bp, its interaction with RPA allows it to unwind substrates as long as 849 bp, the longest substrate tested [36]. Compared to other SSBs such as *E. coli* SSB and T4 gene 32p, hRPA is the most effective in enhancing WRN helicase activity. For example, significantly higher effective concentrations of *E. coli* SSB or T4 gene 32 protein than of hRPA are required to achieve the same extent of helicase stimulation on synthetic oligomer substrates [18]. Moreover, the concentration dependence of stimulation follows a hyperbolic curve in the case of hRPA and a sigmoidal curve in the case of *E. coli* SSB [18]. The sigmoidal curve observed for *E. coli* SSB probably reflects cooperative binding to ssDNA that prevents reannealing of the displaced oligomer while the hyperbolic curve found for hRPA may reflect a non-cooperative, direct interaction between the protein partners rather than the mere coating of the exposed single-stranded [18]. It has been recently reported that RPA alleviates the inhibitory effect of vinylphosphonate internucleotide linkages on DNA unwinding by the WRN helicase, suggesting that RPA may tether the helicase to the DNA substrate at the single-strand/double-strand junction, thus allowing it to cope with rotational rigidity in the DNA template during the unwinding reaction [72]. Although this observation does not provide for a cellular role for WRN, it does provide an insight into the translocation mechanism of the WRN helicase suggesting a combination of base-flipping and phosphodiester interactions for its movement along ssDNA [72]. Similarly, a direct physical and functional interaction between FFA-1, the *Xenopus* homologue of WRN, and RPA has been shown, which stimulates the helicase activity of FFA-1 in a fashion similar to that has been observed with the WRN–RPA interaction [73]. Further, FFA-1 is essential for the formation RPA foci associated with replication [74]. Despite these observations, no specific cellular role can be assigned to the WRN–RPA complex because RPA is involved in all of the major DNA pathways.

5.1.4. FEN-1

An interesting replication protein that interacts with WRN is the FEN-1 protein [75], a 5′-endonuclease/5′–3′-exonuclease that is involved in the maturation of Okazaki fragments during lagging strand DNA replication [76], in long-patch base excision repair (BER) [77], as well as in non-homologous DNA end joining (NHEJ) [78]. WRN and FEN-1 interact through the 144-amino acid RQC domain on the C-terminal region of the WRN protein [75]. While the interaction with FEN-1 does not in any obvious way affect the activity of WRN, WRN greatly stimulates (more than 80-fold) the nucleolytic activity of FEN-1 in a concentration-dependent manner, even if the helicase and exonuclease activities of WRN are abolished [75]. Furthermore, WRN stimulates the cleavage of DNA structures that are poor substrates of FEN-1 alone, suggesting that these two proteins are likely to act together in vivo. Since the C-terminal region of WRN that encompasses the FEN-1-interacting region alone is

able to enhance FEN-1 activity, the helicase activity of WRN seems to be not required for FEN-1 stimulation [75,79]. Recent fluorescence resonance energy transfer (FRET) analyses show that the WRN-FEN-1 complex co-localizes in foci associated with arrested replication forks and further biochemical studies demonstrate that this complex plays a role in the unwinding and degradation of chicken-foot Holliday junction structures associated with regressed replication forks [80]. While collectively these data argue for an important biological function of the WRN-FEN-1 complex, the promiscuous involvement of FEN-1 in DNA replication, repair, and NHEJ pathways unfortunately shadows any insight into the specific role of this complex.

5.2. *Genomic maintenance proteins*

5.2.1. *Ku-DNA–PK complex*

Interestingly, FEN-1 is not the only NHEJ protein that forms a complex with WRN: the physical and functional interaction between the components of the DNA–PK complex, comprising of DNA–PK_{cs} and the Ku70/Ku80 heterodimer, and the WRN protein has been reported by several laboratories. DNA–PK complex participates in repairing double strand breaks caused by physiological oxidative stress, recombination, ionizing radiation, as well as genotoxic chemicals [81]. Affinity binding and co-immunoprecipitation studies revealed a physical interaction between WRN and Ku [82,83] while the use of deletion mutants demonstrated that the N-terminal region of WRN is necessary and sufficient to bind the Ku heterodimer [84]. However, the Ku heterodimer appears to bind both N- and C-terminal domains of WRN under normal conditions [85]. This interaction has no effect on the helicase activity of WRN, but it broadens the exonuclease specificity to hydrolzye blunt ends and protruding 3′ single strands and enhances its processivity [83]. Furthermore, this stimulation can also be observed with the K577M mutant form of the WRN protein [82], which displays no helicase activity, as well as by a recombinant WRN fragment harboring only the exonuclease domain [84], indicating that the helicase activity and the C-terminal Ku-binding domain are not required for this functional interaction.

Recent evidence suggests that WRN can interact directly with DNA–PK_{cs} without the involvement of Ku, and that DNA–PK_{cs} phosphorylates WRN to inhibit its helicase and exonuclease activities, a process that can be reversed by Ku [86]. Since WRN is phosphorylated in vivo in response to bleomycin- or 4-NQO-induced DNA damage [87], and since Ku enables WRN to hydrolyze 8-oxoguanine- and 8-oxoadenine-terminated DNA substrates [48], it is possible that Ku plays a role in the activation of WRN to participate in the removal of certain replication blocks. Additionally, by modulating the exonuclease activity of WRN, DNA–PK complex may limit the processing of DNA ends prior to end joining in NHEJ or other DNA repair processes.

5.2.2. *PARP-1*

Remarkably, it has been shown that WRN and Ku70/80 participate to form a cellular trimeric complex with poly(ADP-ribose) polymerase-1 (PARP-1) [88], a highly conserved nuclear factor implicated in the control of genomic stability and mammalian longevity [89]. PARP-1 participates in one the earliest responses to DNA damage by catalyzing the sequential transfer of ADP-ribose monomers onto a spectrum of nuclear proteins, including itself [90]. Based on in vitro biochemical evidence, as well as affinity purification, immunoblot analysis, and mass spectroscopy experiments, it has been suggested that PARP-1 modulates WRN exonuclease activity (but not helicase activity) [88]. Conversely, unmodified PARP-1 has recently been identified as the most prominent WRN RQC domain binding protein [91].

5.2.3. *DNA polymerase β and APE-1*

There is considerable evidence that WRN participates in base excision repair. In vitro, wild type WRN binds to DNA Polβ and stimulates strand displacement DNA synthesis on a nicked BER intermediate in a reaction requiring the helicase domain of WRN [92]. In addition, recent GST pull-down assays in HeLa nuclear extracts, ELISA assays, immunofluorescence experiments, as well as dot blot assays, demonstrate that WRN forms a stable complex with the major human apurinic/apyrimidinic endonuclease (APE-1) [93], a key player in the early stages of BER. It is proposed that, besides simply bringing WRN to sites of active BER, APE-1 protein prevents the promiscuous unwinding of BER intermediates by WRN until DNA Polβ is recruited for strand displacement synthesis [93].

5.2.4. p53

Recent studies that were initiated on the premises that tumor suppressors may regulate both tumorigenesis and cellular aging and that WRN and p53 may possibly be linked in a common pathway determining cell aging revealed that the key tumor suppressor protein p53 directly associates with the C-terminal portion of WRN, and inhibits its exonuclease activity [94,95]. Wild type p53 attenuates WRN helicase activity and abolishes its ability to unwind synthetic Holliday junctions in vitro; this inhibition is dependent upon the phosphorylation status of key serine residues at the C-terminus of p53 [96]. On a cellular level, the transcription of WRN gene is repressed by p53 [97]. On the other hand, when WRN is artificially overexpressed in normal fibroblasts, p53-dependent transcriptional activity increases and results in the initiation of p53-mediated apoptosis [98].

5.3. Telomeric maintenance and recombination proteins

5.3.1. TRF1 and TRF2

Early statistical evidence indicating an accelerated shortening of telomerase restriction fragments in serially passaged WS cultures [99], together with the indication that the loss of telomeric DNA may determine the onset of replicative senescence (reviewed in [100]), provided an impetus for the investigation of the role of WRN in telomere maintenance. Initial in vitro biochemical experiments demonstrated that WRN helicase/exonuclease was able to disrupt and degrade D-loop substrates [44] that are believed to occur in telomeric regions [101], potentially serving to protect the ends of chromosomes [102]. Additional evidence for the WRN-telomere connection is provided by the participation of the *Saccharomyces cerevisiae* WRN homolog Sgs1p in telomere maintenance in cells lacking telomerase [103]. These cells and their mammalian counterparts prevent the erosion of their telomeres by a telomerase-independent pathway termed ALT (alternative lengthening of telomeres) [104] and are distinguished by the presence of nuclear structures referred to as promyelocytic leukemia (AA-PML) bodies, which contain telomeric repeat DNA, telomeric repeat binding proteins TRF1 and TRF2 protein, and the PML protein [105]. Furthermore, in addition to co-localization, a direct physical and functional interaction between TRF2 and WRN mediated by the highly conserved RQC domain of WRN has been demonstrated [106]. This physical interaction stimulates the WRN helicase activity on short-forked substrates containing telomeric repeats, but shows no effect on the exonuclease activity [106]. Moreover, WRN also binds to TRF1 protein, and the association of TRF2 and TRF1 with the telomeric D-loop limits the extent of WRN exonuclease digestion into the telomeric repeats [107]. This inhibition is independent of the helicase activity of WRN in that the unwinding of D-loops in the presence of RPA is not affected [107]. In marked contrast, Machwe and coworkers have clearly shown that TRF2 specifically facilitates WRN exonuclease activity on substrates containing telomeric repeats that are considerably larger [108]. On an organismal level, a causal link between telomere shortening and the manifestation of Werner syndrome phenotypes has been demonstrated using mouse models where late-generation mice null with respect to both *Wrn* and *Terc* (encoding the telomerase RNA template component) elicit classical WS pathologies accompanied by enhanced telomere dysfunction [109,110]. Finally, recent reports suggest that cells lacking WRN exhibit attrition of telomeres from lagging strand sister chromatids, and that the prevention of the loss of telomeres is WRN helicase-dependent [111]. Collectively, these results argue that WRN may be necessary for efficient replication of G-rich telomeric DNA as well as for the repair and processing of telomeric end structures.

5.3.2. Mre11 complex

Another protein complex that has recently been proposed to cooperate with WRN is the Mre11 complex [112], a three-subunit complex that is composed of Mre11, Rad50, and Nbs1/Xrs2 (reviewed in [113]). Mutations in these genes result in sensitivity to DNA damage, genomic instability, telomere attrition, and aberrant meiosis [113]. WRN co-localizes and physically interacts with this complex at stalled replication forks [114]. Further, it has been shown that WRN interacts with Mre11 via binding to Nbs1 in vivo and in vitro, which results in the promotion of its helicase activity [115]. Moreover, both WRN and Mre11 are phosphorylated in an ATR-dependent manner following replication blockage and co-localization in nuclear foci [116,117]. On a cellular level, mutations that affect the functionality of either WRN or that of the Mre11

complex result in chromosomal breakage during DNA replication and apoptosis following replication arrest [118]. Since the depletion of Mre11 complex by RNAi knockdown does not enhance chromosomal breakage and cell death in WS cells, it is proposed that WRN and Mre11 complex act in a common pathway in response to replication fork arrest [118].

6. In vivo role(s) of WRN

The drive for the study of WRN protein is based on the premise that WS, as a useful model system, can promote the formulation of directed and experimentally tractable mechanistic insights into the process of normal aging as well as age-associated diseases. However, as more and more is uncovered regarding this enticing enzyme, it becomes clear that WRN, with its intricate biochemistry and cell biology, its multiple interacting protein partners and the complex phenotypic manifestations its absence creates, participates in more than a single DNA metabolic pathway. Yet, most lines of evidence presented so far are compatible with an overarching role for WRN in the resolution of alternative DNA structures in a variety of DNA synthetic processes.

WRN is proposed to function during DNA replication to clear the path for the replicative apparatus by resolving alternative DNA structures that would otherwise impede the progression of the replication fork. The dual helicase/exonuclease functionality of WRN is exceptionally well suited for the processing of non-canonical DNA structures. The interactions of WRN with DNA Polδ, its association with topoisomerase I and PCNA in the 17S replication complex, as well as its physical and functional interactions with RPA and FEN-1 give further credence to the argument that WRN is involved in replication. The fact that WS cells display a prolonged S-phase strengthens this argument. However, since many of these proteins are also involved in DNA repair, an exclusively replication-specific role to WRN cannot be assigned.

In addition to the proteins with dual roles in DNA replication and repair that are mentioned above, WRN has been shown to interact with repair proteins such as DNA Polβ, Ku and its associated DNA–PK_{cs}, PARP-1 and APE1. On a cellular level, WS cells accumulate chromosomal rearrangements and somatic mutations at an increased rate in an age-dependent manner. Additionally, these cells are hypersensitive to some but not all types of DNA damaging agents, and WS lymphoblastoid cell lines show reduced levels of gene-specific and strand specific repair of UV damage. Furthermore, in concordance with the ability of WRN to resolve three- and four-way junctions, WS cells are impaired in their capacity to resolve mitotic recombination products. Then again, in addition to repair, these structural intermediates arise in a variety of DNA metabolic processes such as replication, repair, and recombination.

Finally, the increased loss of telomeres in WS cells, the correlation between aging and telomeric attrition, as well as the high specificity of WRN for G-rich alternative DNA structures found in telomeres, suggest a role for WRN in telomere maintenance. Further substantiating this link is the association of WRN with telomere repeat binding factors TRF1 and TRF2.

7. Conclusion

Werner syndrome hides important clues to the biology of aging and age-associated diseases. So far, our detailed analyses of the biochemistry of the encoded protein has defined its function as a helicase and exonuclease but has not established its precise role in in vivo DNA transactions. WRN could be a sticky protein that associates with a wide variety of partners, each of which appears to be involved in multiple DNA synthetic processes. Again, these associations have so far failed to yield definitive mechanistic insights into cellular pathways. Functional interactions between WRN and associated proteins are likely to be the most definitive in guiding our understanding, particularly those interactions that involve enhancement in enzyme activities. Considering the power of *E. coli* genetics and the homologies between WRN and RecQ, our understanding of Werner syndrome may ultimately require our understanding of RecQ helicase, as Phil Hanawalt so prognosticated.

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