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Abstract: Reactive oxygen species constantly generated as by-products of cellular metabolism readily attack genomic DNA creating mutagenic lesions such as 7,8-dihydro-8-oxo-guanine (8-oxo-G) that promote aging. 8-oxo-G:A mispairs arising during DNA replication are eliminated by base excision repair initiated by the MutY DNA glycosylase homologue (MUTYH). Here, by using formaldehyde crosslinking in mammalian cell extracts, we demonstrate that the WRN helicase/exonuclease defective in the premature aging disorder Werner syndrome (WS) is recruited to DNA duplex containing an 8-oxo-G:A mispair in a manner dependent on DNA polymerase ￿ (Pol ￿) that catalyzes accurate DNA synthesis over 8-oxo-G. Similarly, by immunofluorescence, we show that Pol ￿ is required for accumulation of WRN at sites of 8-oxo-G lesions in human cells. Moreover, we show that nuclear focus formation of WRN and Pol ￿ induced by oxidative stress is dependent on ongoing DNA replication and on the presence of MUTYH. Cell viability assays reveal that depletion of MUTYH suppresses the hypersensitivity of cells lacking WRN and/or Pol ￿ to oxidative stress. Biochemical studies demonstrate that WRN binds to the catalytic domain of Pol ￿ and specifically stimulates DNA gap filling by Pol ￿ over 8-oxo-G followed by strand displacement synthesis. Our results suggest that WRN promotes long-patch DNA repair synthesis by Pol ￿ during MUTYH-initiated repair of 8-oxo-G:A mispairs.

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Involvement of Werner syndrome protein in MUTYH-mediated repair of oxidative DNA damage

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ABSTRACT

Reactive oxygen species constantly generated as by-products of cellular metabolism readily attack genomic DNA creating mutagenic lesions such as 7,8-dihydro-8-oxo-guanine (8-oxo-G) that promote aging. 8-oxo-G:A mispairs arising during DNA replication are eliminated by base excision repair initiated by the MutY DNA glycosylase homologue (MUTYH). Here, by using formaldehyde crosslinking in mammalian cell extracts, we demonstrate that the WRN helicase/exonuclease defective in the premature aging disorder Werner syndrome (WS) is recruited to DNA duplex containing an 8-oxo-G:A mispair in a manner dependent on DNA polymerase \( j \) (Pol \( j \)) that catalyzes accurate DNA synthesis over 8-oxo-G. Similarly, by immunofluorescence, we show that Pol \( j \) is required for accumulation of WRN at sites of 8-oxo-G lesions in human cells. Moreover, we show that nuclear focus formation of WRN and Pol \( j \) induced by oxidative stress is dependent on ongoing DNA replication and on the presence of MUTYH. Cell viability assays reveal that depletion of MUTYH suppresses the hypersensitivity of cells lacking WRN and/or Pol \( j \) to oxidative stress. Biochemical studies demonstrate that WRN binds to the catalytic domain of Pol \( j \) and specifically stimulates DNA gap filling by Pol \( j \) over 8-oxo-G followed by strand displacement synthesis. Our results suggest that WRN promotes long-patch DNA repair synthesis by Pol \( j \) during MUTYH-initiated repair of 8-oxo-G:A mispairs.

INTRODUCTION

Reactive oxygen species constantly produced in living organisms as byproducts of normal cellular metabolism or as a consequence of environmental exposure to various physical and chemical agents can generate a variety of oxidized DNA bases that are highly mutagenic and hence compromise genomic stability, promoting aging and carcinogenesis (1–4). One of the most frequent oxidative lesions is 7,8-dihydro-8-oxo-guanine (8-oxo-G) with a steady-state level of about 10^3 lesions per cell in normal tissue (5). Replication of genomic DNA containing 8-oxo-G lesions frequently leads to the formation of 8-oxo-G:A mispairs, giving rise to a G:C to T:A transversion mutations (6). Interestingly, these transversions are among the predominant somatic mutations found in lung, breast, ovarian, gastric and colorectal cancers, suggesting that a failure to eliminate 8-oxo-G lesions can initiate tumorigenesis and drive tumor progression (7).

Oxidized base lesions are primarily eliminated by the base excision repair (BER) system (8). In mammalian cells, the repair of 8-oxo-G:A mispairs is achieved via two BER events that occur sequentially on the two DNA strands (9). The first event is initiated by excision of the mispaired A residue by the MutY glycosylase homologue (MUTYH) in a reaction coordinated by proliferating cell nuclear antigen (PCNA) (10–12). This is followed by cleavage of the apurinic site (AP) by the AP endonuclease 1 (APE1), creating a DNA gap with a 3'-OH moiety (12,13). PCNA and replication protein A (RPA) then govern the bypass of the 8-oxo-G lesion by the DNA polymerase \( \lambda \) (Pol \( \lambda \)), which in the presence of these two auxiliary factors preferentially incorporates dCTP opposite the lesion (12,14,15). Following lesion bypass, RPA dissociates and PCNA recruits flap
endonuclease 1 (FEN1) to remove the 5’-single-stranded DNA (ssDNA) flap resulting from the limited strand displacement synthesis by Polδ (12). Finally, DNA ligase I interacts with PCNA loaded on the nick arising from FEN1 cleavage and seals it, creating the substrate for a second BER event, which leads to the elimination of the 8-oxo-G lesion (12). 8-oxo-G paired with C is predominantly excised by the OGG1 glycosylase in a short patch BER reaction in which Polβ fills the DNA gap and the DNA ligase III/XRCC1 complex restores the continuity of the damaged DNA strand (8).

Werner syndrome (WS) is an autosomal recessive disorder characterized by premature aging, cancer predisposition and genomic instability (16). It is caused by mutations in the WRN gene which encodes a multifunctional protein (WRN) possessing 3’–5’ DNA helicase and 3’–5’ exonuclease activities (16). Interestingly, WRN-deficient cells accumulate 8-oxo-G lesions at a much higher rate than WRN-proficient cells (17,18). However, the molecular basis of this phenomenon is not known. Here we present several lines of evidence suggesting that WRN cooperates with Polδ to carry out long-patch DNA repair synthesis during MUTYH-initiated repair of 8-oxo-G:A mispairs. Loss of such an activity might explain many cellular phenotypes associated with WS including accumulation of oxidative DNA lesions, accelerated telomere attrition and genomic instability.

MATERIALS AND METHODS

Antibodies and purified proteins
All primary antibodies used for immunofluorescence staining and immunoblotting are described in Supplementary Materials and Methods. Recombinant human Polδ protein was expressed and purified as previously described (19). His-tagged recombinant human Polδ fragments were purified on Ni-NTA agarose (Invitrogen) as recommended by the manufacturer. Recombinant human WRN protein and its mutants were produced and purified as previously described (20). These protein preparations had a purity of >95% (Supplementary Figure S1A) and did not contain any contaminating DNA polymerase activity (Supplementary Figure S1C). Purified wild-type WRN could unwind efficiently a forked DNA duplex (Supplementary Figure S1B). RECQ5 and BLM proteins were purified as previously described (21,22).

Cell culture experiments
All cell lines (HeLa, U2OS, HEK293, MEFs, MRC5) used in this study were maintained in DMEM (Gibco) supplemented with 10% fetal calf serum (Gibco) and streptomycin/penicillin (100 U/ml). Where required, H2O2 (Sigma) was added to cell cultures to a final concentration as indicated. Hydroxyurea (Sigma) was used at a concentration of 2 mM to synchronize cells at G1/S boundary. To arrest DNA replication, cells were treated with aphidicolin (Sigma) at a concentration of 1 μg/ml. Transfection of siRNA oligonucleotides was carried out using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer’s instructions. Cells were analyzed 72 h after siRNA transfection. The sequences of the siRNA oligonucleotides used in this study are indicated in Supplementary Materials and Methods.

Crosslinking assay
Formaldehyde crosslinking assays with cell extracts and hairpin oligonucleotide substrates attached to streptavidin magnetic beads (Invitrogen) were performed as described previously (12).

Chromatin-binding assay
Chromatin fractionation was done as previously described (23). Briefly, to obtain cytoplasmic fraction, HEK293T cells were resuspended in buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 1 mg/ml digitonin, Roche complete protease inhibitor cocktail) and incubated on ice for 5 min. Nuclei were collected by centrifugation at 1500 g for 4 min, washed once with buffer A, resuspended in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, Roche complete protease inhibitor cocktail) and incubated on ice for 15 min. Chromatin was separated from nucleoplasmic fraction by low speed centrifugation at 2000 g for 4 min, washed twice in buffer B, resuspended in SDS loading buffer and sonicated. Bound proteins were analyzed by western blotting.

Immunofluorescence assays
Cells cultured on glass coverslips were fixed with 3.7% formaldehyde for 10 min at room temperature (RT) and subsequently permeabilized by soaking in 0.2% (v/v) Triton X-100 for 5 min at RT. After blocking in PBS containing 5 mg/ml BSA for 30 min at RT, the fixed cells were incubated overnight at 4°C with appropriate primary antibodies. The slides were washed with PBS and incubated for 1.5 h at RT with secondary antibodies diluted in blocking solution: fluorescein isothiocyanate (FITC) conjugated sheep anti-rabbit IgG (Sigma; 1:700) and Texas Red-conjugated donkey anti-mouse IgG (Jackson Immunoresearch, 1:200). After washing with PBS, coverslips were mounted on Vectashield (Vector Laboratories) and images were captured on an Olympus IX81 fluorescence microscope. At least 100 nuclei were analyzed in each of three independent experiments. For simultaneous detection of WRN and 8-oxo-G, cells were fixed and sequentially incubated with rabbit polyclonal anti-WRN antibody and anti-rabbit FITC-conjugated secondary antibody. Stained cells were then fixed with 100% cold methanol for 30 min at −20°C and immersed in 100% cold acetone for 30 s. After washing, the fixed cells were treated with 2M HCl for 30 min to denature the DNA and then neutralized with 0.1 M borate buffer (pH 8.5). After washing and blocking, cells were stained with mouse monoclonal anti-8-oxo-G (IgM) antibody (1:100) followed by Texas Red-conjugated donkey anti-mouse IgM secondary antibody (Jackson Immunoresearch, 1:150). After washing, coverslips were mounted and analyzed as described above. The same procedure was used for simultaneous detection of Polδ and 8-oxo-G lesion.
Immunoprecipitation assays

Total cell extract preparation and immunoprecipitation (IP) were carried out as described previously (24). For IP of purified recombinant proteins, a mixture of equal amounts of WRN (300 ng) and Pol<sub>C14</sub> (300 ng) was incubated for 2 h at 4°C and then added to Protein A/ G-agarose beads (20 μl) coated with rabbit polyclonal anti-WRN IgGs (2 μg). After incubation for 2 h at 4°C, the beads were washed and subjected to western blot analysis. All the IP reactions were conducted in the presence of DNase I (Roche) to exclude the possibility of protein–protein interaction mediated through DNA.

Gap-filling assay

Annealing of a 72-mer containing 8-oxo-G lesion (or a normal G) with the 5’-[32P]-labeled 39-mer primer created a primer/template substrate with the lesion (or a normal G) at the +1 position relative to single-strand/double-strand junction. Annealing of a 32-mer oligonucleotide to the region 3’ of the lesion site in this structure yielded a duplex containing a one-nucleotide (1-nt) gap opposite 8-oxo-G (or normal G). The 32-mer oligonucleotide was phosphorylated by T4 polynucleotide kinase (NEB) prior to annealing. The reaction mixtures (10 μl) contained 50 mM Tris–HCl (pH 7.5), 2 mM DTT, 0.25 mg/ml BSA, 10 μM dNTP, 1 mM MgCl2 and 10 fmol of the 5’-[32P]-labeled DNA substrate. Concentrations of Pol<sub>C0</sub> and WRN are indicated in figures and figure legends. Reactions were carried out at 37°C for 10 min. Reaction mixtures were separated on a denaturing urea-polyacrylamide gel and radiolabeled DNA species were visualized by phosphorimaging on a Typhoon 9400. Gel images were quantified using ImageQuant software.

Biotin pull-down assay

Pull-down assays using biotinylated gapped DNA duplexes with or without 8-oxo-G lesion were performed as described previously (15).

Cell viability assay

Cell viability assays were done as previously described (25). Briefly, HeLa cells were seeded at a confluency of 25% in cell culture plates containing complete medium (DMEM, 10% FCS and penicillin/streptomycin). After 24 h, cells were transfected with the appropriate siRNAs. 2 days after transfection, siRNA-treated cells were harvested and seeded in a 96-well plate at a density of 2500 cells/well in a volume of 100 μl of complete medium. After 24 h, cells were treated with different concentrations of H<sub>2</sub>O<sub>2</sub> (Sigma) ranging from 0 to 640 μM. Experiments were carried out in hexaplicates for each H<sub>2</sub>O<sub>2</sub> concentration. 2 h after H<sub>2</sub>O<sub>2</sub> addition, cells were washed twice with PBS, and allowed to grow in complete medium. After 2 days, a mixture of resazurin and complete medium (ratio of 1:10; 100 μl) was added to individual wells. Cell viability was measured after 4 h of incubation using a SpectraMax reader M5 (Molecular Devices). The percentage of viable cells was calculated relative to mock treated cells and plotted using GraphPad Prism as mean ± SD. Data from at least two independent experiments were plotted.

RESULTS

WRN is involved in the processing of 8-oxo-G:A mispairs in mammalian cell extracts

To investigate whether WRN is involved in the repair of 8-oxo-G:A mispairs, we employed a previously established assay using formaldehyde crosslinking in human cell extracts to monitor the recruitment of BER proteins to damaged DNA (26). A 3’-biotinylated hairpin loop oligoduplex (27 bp) containing a single 8-oxo-G:A mispair was incubated with HeLa whole-cell extract in the presence of Mg<sup>2+</sup> to initiate repair (12). As a control reaction, the corresponding lesion-free substrate was also tested. At different time points, the reactions were stopped by the addition of formaldehyde and cross-linked DNA–protein complexes, isolated using streptavidin magnetic beads, were analyzed by western blotting. We observed a rapid, damage-specific recruitment of WRN to the DNA substrate (Figure 1A). A robust damage-specific recruitment of Pol<sub>B</sub> was also detected as previously reported (12). To address whether the recruitment of WRN to the hairpin duplex containing 8-oxo-G:A mispair was dependent on Pol<sub>B</sub>, whole-cell extracts from Pol<sub>B</sub><sup>−/−</sup> and Pol<sub>B</sub><sup>+/+</sup> MEFs is shown in Figure 4B (right panel).
mouse embryonic fibroblasts (MEFs) were prepared and subjected to formaldehyde crosslinking assay. We found that in the absence of Polδ, WRN was not cross-linked to the hairpin substrate, suggesting that Polδ mediates the recruitment of WRN to the sites of repair of 8-oxo-G:A mispairs (Figure 1B). This is further supported by the finding that complementation of Polδ−/− MEFs with human Polδ cDNA lead to a restoration of WRN recruitment to the hairpin duplex in formaldehyde crosslinking assay (Supplementary Figure S2). These results provide a strong evidence for the involvement of WRN in the repair of 8-oxo-G:A mispairs.

WRN accumulates at sites of 8-oxo-G lesions during S-phase in a manner dependent on DNA replication and the presence of Polδ.

To investigate whether WRN is recruited to sites of oxidative DNA damage in vivo, we first performed sub-cellular fractionation to analyze chromatin binding of WRN in HEK293T cells prior to and after treatment with H2O2. We found that at 2.5h after H2O2 addition, the amount of WRN bound to chromatin substantially increased as compared to non-treated cells (Figure 2). Likewise, we observed that H2O2 treatment promoted chromatin binding of Polδ (Figure 2).

Next, we used indirect immunofluorescence technique to monitor the spatial distribution of WRN and Polδ in U2OS cells after H2O2 treatment. In agreement with previous reports, WRN was observed to localize to the nucleolus in the majority of non-treated cells, whereas Polδ showed a dispersed nuclear staining (Figure 3A, top row). Upon treatment of cells with 500 μM H2O2 for 2h, WRN and Polδ each formed ≥10 distinct foci per nucleus in 45 and 65% of cells, respectively. Approximately in 35% of these cells, more than 75% of WRN foci co-localized with Polδ foci (Figure 3A, bottom row, and Supplementary Table S1). Notably, the frequency of nuclei with co-localizing foci of WRN and Polδ increased substantially when H2O2 was added to cells that had been released from G1/S blockade, suggesting that the re-localization of these proteins induced by oxidative stress is specific to S-phase cells (Figure 3B, Supplementary Figure S3). In accordance with this assumption, we found that after H2O2 treatment, WRN and Polδ formed foci only in cells expressing cyclin A (Supplementary Figure S4). To substantiate these findings, cells were treated with aphidicolin for 20 min prior to addition of H2O2 to inhibit DNA replication. We found that aphidicolin impaired the formation of WRN and Polδ foci after oxidative stress, suggesting that WRN and Polδ are recruited to site of oxidative DNA damage in a manner dependent on DNA replication that can give rise to 8-oxo-G:A mispairs (Figure 3C).

To prove that WRN and Polδ co-localized at sites of oxidative DNA damage, untreated and H2O2-treated cells were co-immunostained either for visualization of WRN and 8-oxo-G or for visualization of Polδ and 8-oxo-G. The results indicated that WRN and Polδ co-localized with sites of 8-oxo-G lesions in ~40 and ~50% of cells, respectively (Figure 4A, Supplementary Tables S2 and S3). To explore the mechanism underlying the recruitment of WRN to sites of 8-oxo-G lesions in vivo, we analyzed by immunofluorescence the spatial distribution of WRN in Polδ+/+ and Polδ−/− MEFs prior to and after treatment with H2O2. The results demonstrated that genetic ablation of Polδ impaired the re-localization of WRN to sites of 8-oxo-G lesions (Figure 4B and Supplementary Figure S5), which is consistent with the data from the crosslinking experiments described above (Figure 1B). We also tested the effect of WRN depletion on the accumulation of Polδ at sites of 8-oxo-G lesions in U2OS cells. We found that lack of WRN did not abolish Polδ focus formation after oxidative stress (Figure 4C). Instead, a small increase in the frequency of cells with co-localizing foci of 8-oxo-G and Polδ was observed in WRN-deficient cells relative to WRN-proficient cells both in the presence and in the absence of H2O2 (Figure 4C). This is in agreement with the previous finding of elevated levels of spontaneous oxidative DNA damage in WRN-deficient cells (17). Collectively, the results described above provide strong evidence that WRN is recruited to sites of 8-oxo-G lesions in a DNA replication- and Polδ-dependent manner.

WRN is required for repair of 8-oxo-G:A mispairs in human cells

Earlier studies revealed that Polδ−/− MEFs were more sensitive to oxidative stress than normal MEFs (27). To prove that WRN functions in repair of oxidative DNA damage in conjunction with Polδ, we employed resazurin-based cell viability assay to test the effect of single and combined depletions of WRN and Polδ on sensitivity of HeLa cells to H2O2 treatment. In agreement with the previous report, we found that Polδ-depleted cells displayed a higher sensitivity to H2O2 compared to mock-depleted cells (Figure 5A and E). WRN-depleted
cells exhibited a similar degree of sensitivity to H$_2$O$_2$ as Pol$\lambda$/C21-depleted cells (Figure 5A and E). Co-depletion of WRN and Pol$\lambda$/C21 did not result in a further decrease in H$_2$O$_2$ sensitivity compared to singly-depleted cells, suggesting that WRN and Pol$\lambda$/C21 act in the same pathway of oxidative DNA damage repair (Figure 5A and E).

To substantiate the above findings, we investigated whether depletion of WRN and Pol$\lambda$/C21 caused cell death in response to oxidative stress. Using Annexin V-binding assay, we found that lack of either protein leads to a significant increase in the frequency of dead cells after H$_2$O$_2$ treatment as compared to mock-depleted cells (Supplementary Figure S6). Again, no additive effects on cell death were observed upon co-depletion of WRN and Pol$\lambda$, confirming that these proteins act epistatically to protect cells against oxidative DNA damage (Supplementary Figure S6).

We reasoned that the hypersensitivity of cells deficient for WRN and Pol$\lambda$ to H$_2$O$_2$ was a consequence of accumulation of ssDNA breaks resulting from MUTYH-mediated processing of 8-oxo-G:A mispairs. Therefore, we tested whether the defect conferred by WRN/Pol$\lambda$ deficiency could be rescued by the elimination of MUTYH. Indeed, we found that depletion of MUTYH from HeLa cells lacking WRN and/or Pol$\lambda$ restored the cellular sensitivity to H$_2$O$_2$ to control levels (Figure 5B–E and Supplementary Figure S6). Depletion of MUTYH alone did not significantly alter H$_2$O$_2$ sensitivity of cells proficient for WRN and Pol$\lambda$ (Figure 5, compare panels B–D with panel A). Together, these data provide strong evidence that WRN and Pol$\lambda$ are required for repair of 8-oxo-G:A mispairs initiated by the MUTYH DNA glycosylase.

To obtain further evidence for the above statement, we tested the effect of MUTYH depletion on the re-localization of WRN and Pol$\lambda$ to nuclear foci in response to oxidative stress. We found that lack of MUTYH dramatically impaired the nuclear focus formation of both WRN and Pol$\lambda$ in U2OS cells after H$_2$O$_2$ treatment, suggesting that these foci represent sites of repair of 8-oxo-G:A mispairs (Figure 5F).

**WRN physically interacts with Pol$\lambda$.**

To investigate whether WRN and Pol$\lambda$ interact physically, an extract of HEK293 cells was subjected to IP with anti-WRN antibody. We found that Pol$\lambda$ co-immunoprecipitated with WRN, while it was not present in an immunoprecipitate obtained with control IgG, suggesting that these proteins formed a stable complex in vivo (Figure 6A). Interestingly, the level of WRN–Pol$\lambda$
complex in the extract from cells treated with H2O2 was notably higher compared to that in the extract from non-treated cells, suggesting that the formation of WRN–Polε complex in cells might be stimulated by oxidative DNA damage (Figure 6A, compare lanes 3 and 4). We also performed co-IP experiments with a mixture of purified recombinant proteins and found that WRN and Polε co-precipitated with anti-WRN antibody, but not with control IgG, indicating that WRN and Polε interact directly (Figure 6B).

To map the WRN-binding site on Polε, co-IP experiments were performed with different deletion variants of Polε (Figure 6C). A stable interaction of WRN with Polε1–244, but not with Polε1–244 and Polε133–244 was observed, suggesting that WRN binds to the catalytic core domain of Polε (Figure 6C). We also used GST pull-down assay to map the region of WRN that binds to Polε. We found that Polε bound well to GST–WRN949–1432 and GST–WRN500–946, but not to GST–WRN51–499 or to GST alone (Supplementary Figure S7). These results indicated that Polε interacted with the helicase domain of WRN (amino acids 500–946) as well as with the C-terminal region of WRN, which contains winged-helix domain, a binding site of a number of other proteins shown to interact with WRN (28).

WRN specifically stimulates the bypass of 8-oxo-G by Polε on gapped DNA duplex

In order to gain insight into the role of WRN in the repair of 8-oxo-G:A mispairs, we tested its effect on the gap-filling activity of Polε using a synthetic DNA duplex containing a centrally located 1-nit gap opposite to 8-oxo-G (Figure 7A). We found that WRN significantly stimulated Polε to fill the gap opposite the 8-oxo-G lesion and to carry out strand displacement synthesis (Figure 7A). Quantification of gel images from these experiments indicated that WRN increased the extent of Polε reaction about 3-fold (Figure 7B). In contrast, WRN did not stimulate the gap-filling activity of Polε on DNA substrate containing a normal G opposite the gap (Figure 7A, lanes 3–6). Moreover, stimulation of Polε was not observed with other RecQ helicases such as BLM and RECQ5, and WRN did not enhance 8-oxo-G bypass by Polβ on gapped DNA duplex (Figure 7A, lanes 11, 15 and 16, and Supplementary Figure S8). These data indicate that WRN stimulates specifically Polε-mediated bypass of 8-oxo-G on gapped DNA duplex.

We also tested the effect of WRN on 8-oxo-G bypass by Polε following MUTYH/APE1-mediated processing of an 8-oxo-G:A mispair in a 100-bp DNA duplex (12). We found that WRN significantly enhanced the trans-lesion synthesis mediated by Polε in this system (~1.7-fold), providing evidence that WRN promotes lesion bypass by Polε during MUTYH-initiated repair of 8-oxo-G:A mispairs (Supplementary Figure S9).

To substantiate these findings, we tested binding of WRN to the 8-oxo-G-containing and lesion-free gapped DNA substrates in the presence and in the absence of Polε. Reactions also contained dCTP to allow Polε to
incorporate 2 nt. We found that Pol₂ was bound to both DNA substrates irrespective of the presence or absence of WRN, showing a higher affinity for the 8-oxo-G substrate (Figure 7C, compare lanes 4, 5, 8 and 9). Importantly, WRN did not bind to the gapped duplex containing normal G opposite the gap even in the presence of Pol₂, while it did bind efficiently to the 8-oxo-G-containing gapped duplex providing that Pol₂ was present (Figure 7C, lanes 3, 5, 7 and 9).

To address whether the enzymatic activities of WRN were required for its stimulatory effect on DNA synthesis by Pol₂, we used previously established mutants of WRN defective either in the exonuclease (E84A) or in the helicase (K577M) activity (29,30). We found that both mutants stimulated translesion synthesis by Pol₂ on the 8-oxo-G-containing gapped duplex to a similar degree as wild-type WRN (Figure 7D). In agreement with this result, we found that expression of either WRN mutant in WS fibroblasts restored oxidative stress tolerance to the wild-type level, suggesting that WRN might act non-catalytically in repair of oxidative DNA damage in vivo (Supplementary Figure S10).

RPA and PCNA were shown to promote accurate bypass of 8-oxo-G by Pol₂, by repressing the formation of...
of 8-oxo-G:A mispair and stimulating the formation of 8-oxo-G:C base pair (12). Therefore, we finally investigated the effect of WRN on incorporation by Pol/ polymers opposite 8-oxo-G. We found that WRN stimulated the incorporation of both dCTP and dATP opposite 8-oxo-G on gapped DNA duplex, indicating that WRN does not improve the fidelity of 8-oxo-G bypass by Pol/ per se (Supplementary Figure S11).

**DISCUSSION**

The WRN protein has been implicated in a number of cellular processes including telomere maintenance, homologous recombination, replication fork recovery and repair of alkylation DNA damage (31–35). There are also reports suggesting a role for WRN in repair of oxidative DNA damage, but the underlying mechanism is not clear (17,18). Here we provide several lines of evidence suggesting that WRN is required for the correction of 8-oxo-G:A mispairs, which is mediated by the long patch BER pathway initiated by the MUTYH DNA glycosylase (12). Firstly, we found that WRN accumulated at sites of 8-oxo-G lesions in a manner dependent on Pol, an X-family DNA polymerase that mediates translesion synthesis during MUTYH-initiated repair of 8-oxo-G:A mispairs (12). Nuclear focus formation of WRN and Pol after exposure of cells to oxidative stress was found to be dependent on DNA replication and on the presence of MUTYH, strongly suggesting that WRN and Pol accumulate at sites of 8-oxo-G:A mispairs.

Secondly, we found that depletion of WRN from human cells increased their sensitivity to H2O2 to a level similar to that displayed by cells lacking Pol. Together with the finding that co-depletion of WRN and Pol did not have an additive effect on the cellular sensitivity to oxidative stress, these data demonstrate that WRN and Pol act in the same DNA repair pathway. Most importantly, the hypersensitivity of WRN- and Pol-deficient cells to H2O2 was completely suppressed by depletion of MUTYH, strongly suggesting that WRN and Pol are involved in the repair of 8-oxo-G:A mispairs initiated by the MUTYH DNA glycosylase. One can imagine a scenario where in absence of Pol and/or WRN, ssDNA breaks resulting from the processing of 8-oxo-G:A mispairs by MUTYH and APE1 are converted to DNA double-strand breaks in the next round of DNA replication, leading to cell cycle arrest followed by DNA repair or cell death (Supplementary Figure S12).
Finally, we show that WRN forms a stable complex with Pol \( \lambda \) in vivo and in vitro and stimulates specifically the bypass of 8-oxo-G by Pol \( \lambda \) on a gapped DNA duplex resembling the DNA intermediate generated during MUTYH-initiated repair of 8-oxo-G:A mispairs. Moreover, we show that WRN stimulates 8-oxo-G bypass by Pol \( \lambda \) following MUTYH/APEI-mediated processing of 8-oxo-G:A mispairs in vitro. WRN stimulated not only DNA gap filling by Pol \( \lambda \) opposite 8-oxo-G, but it also enhanced the subsequent strand displacement synthesis by Pol \( \lambda \). Therefore, it appears that the role of WRN in MUTYH-initiated repair of 8-oxo-G:A mispair is to promote this pathway in direction of long-patch BER. It is known that DNA ligase I is not able to efficiently ligate a DNA substrate containing a nick in the vicinity of 8-oxo-G:C pair, but is able to seal a nick located 1 nt downstream of the correctly incorporated C (12). Thus stimulation of Pol \( \lambda \)-mediated strand displacement synthesis would enable more efficient completion of accurate MUTYH-initiated BER. Interestingly, previous studies have shown that WRN physically interacts with FEN1 and strongly stimulates FEN1-catalyzed cleavage of 5'-flap substrates (36). Such an activity is essential to generate a ligatable 5'-P end following strand displacement synthesis by Pol \( \lambda \) during the MUTYH-initiated repair of 8-oxo-G:A mispairs. Thus, it is possible that, in addition to stimulation of Pol \( \lambda \)-mediated long-patch DNA repair synthesis, WRN promotes the endonucleolytic cleavage by FEN1 in this BER pathway.

Our study has shown that the helicase and exonuclease activities of WRN are dispensable for Pol \( \lambda \) stimulation by WRN. Similarly, the functional interaction of WRN with FEN1 was independent of the WRN catalytic functions (36). These findings suggested that the stimulatory effect of WRN on these enzymes stems from direct protein–protein interaction. It is possible that physical interaction...
between WRN and Polζ triggers a conformational change in the polymerase, which alters its catalytic properties. Of note, WRN was shown to stimulate DNA synthesis by the Y-family translesion polymerases η, τ and κ by increasing the reaction rate (37).

We have also obtained evidence suggesting that the catalytic activities of WRN are not essential for repair of oxidative DNA damage in the cell. This notion is consistent with the fact that there are no case reports of WS patients carrying loss-of-function mutations in the exonuclease or in the helicase domains of WRN. All WS-causing mutations reported thus far result either in the elimination of the nuclear localization signal located at the C-terminus of WRN or lead to an unstable protein (38).

Importantly, WRN stimulated DNA gap filling and strand displacement synthesis by Polζ only if the DNA substrate contained an 8-oxo-G lesion opposite the gap. Accordingly, although Polζ could bind to both lesion-free and 8-oxo-G-containing substrates, WRN was bound only to the latter substrate in a manner dependent on Polζ. Thus, it is possible that binding of Polζ to the lesion generates a particular structure either on the DNA or in Polζ itself, allowing Polζ-WRN interaction. Our finding of direct interaction of WRN with the catalytic domain of Polζ suggest that a lesion-induced structural change in Polζ leading to the exposure of the WRN interaction site is the more likely scenario. In the complex of Polζ with lesion-free gapped duplex, the WRN interaction site on Polζ might be hindered by DNA. Future structural studies will be needed to shed more light on this issue.

Cells derived from individuals suffering from WS show premature senescence and accelerated telomere shortening, which explains the early onset of aging manifested in these patients and suggest that WRN plays a role in telomere metabolism (16). Evidence indicates that genomic instability in WS cells depends directly on telomere dysfunction, suggesting that the primary cause of high cancer incidence in WS is a breakdown in telomere integrity (39). It is well known that oxidative stress accelerates telomere shortening and shortens replicative lifespan of cultured human primary fibroblasts (40).

A recent study has revealed that disruption of the mouse Ogg1 gene is associated with accumulation of oxidative guanine lesions in telomeres and increased telomere attrition upon oxidative stress, suggesting that oxidative stress compromises telomere integrity through induction of oxidative base damage (41). Thus, based on the earlier finding that WRN deficiency leads to accumulation of 8-oxo-G lesions in the genomic DNA (17,18) and our data implicating WRN in the repair of 8-oxo-G:A mispairs, it is tempting to speculate that WS phenotypes arise from a defect in repair of oxidative DNA base damage in telomeres. CO-FISH analysis of telomeric repeats revealed that the oxidative stress-induced telomere shortening in Ogg1−/− primary MEFs most frequently arises from single-strand breaks in the G-strand, which compromise telomere lagging strand synthesis (41). Interestingly, preferential telomere lagging strand loss was also demonstrated in cells lacking WRN or FEN1 (31,42). Thus, it is possible that the accelerated telomere shortening observed in these cells stems from persistence of 8-oxo-G lesions within the telomeric G-strand due to defective repair of 8-oxo-G:A mispairs. Therefore, it will be interesting to evaluate the role of the other enzymes acting in this BER pathway, such as MUTYH or Polζ, in promoting telomere integrity upon oxidative stress. Such studies will shed more light on how oxidative stress contributes to aging and cancer development.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–3, Supplementary Figures 1–12, Supplementary Materials and Methods and Supplementary References [12,22,24,43–45].

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