The Human Werner Syndrome Protein Stimulates Repair of Oxidative DNA Base Damage by the DNA Glycosylase NEIL1

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The mammalian DNA glycosylase, NEIL1, specific for repair of oxidatively damaged bases in the genome via the base excision repair pathway, is activated by reactive oxygen species and prevents toxicity due to radiation. We show here that the Werner syndrome protein (WRN), a member of the RecQ family of DNA helicases, associates with NEIL1 in the early damage-sensing step of base excision repair. WRN stimulates NEIL1 in excision of oxidative lesions from bubble DNA substrates. The binary interaction between NEIL1 and WRN (Kd = 60 nM) involves C-terminal residues 288–349 of NEIL1 and the RecQ C-terminal (RQC) region of WRN, and is independent of the helicase activity WRN. Exposure to oxidative stresses enhances the NEIL-WRN association concomitant with their strong nuclear co-localization. WRN-depleted cells accumulate some prototypical oxidized bases (e.g. 8-oxoguanine, FapyG, and FapyA) indicating a physiological function of WRN in oxidative damage repair in mammalian genomes. Interestingly, WRN deficiency does not have an additive effect on in vivo damage accumulation in NEIL1 knockdown cells suggesting that WRN participates in the same repair pathway as NEIL1.

Oxidative damage to the mammalian genome represents the most pervasive genotoxic insult and includes a plethora of damaged bases, apurinic/apyrimidinic (AP) sites and DNA strand breaks (1, 2). Such damage has been etiologically linked to a variety of pathological states, including sporadic cancers and aging (3, 4). Most oxidized bases in DNA are repaired via the base excision repair (BER) pathway (5–7). Mammalian BER consists of two sub-pathways: short-patch (SP-BER) and long-patch (LP-BER) (8). Both pathways are initiated with excision of the damaged base by a DNA glycosylase, followed by cleavage of the DNA backbone by AP endonuclease (APE1) or by the intrinsic AP lyase activity in the case of oxidized base-specific glycosylases (6). The strand cleavage by the APE1 generates 3’-OH and 5’-deoxyribose phosphate termini, whereas the AP lyases produce 3’-blocking phosphoribose or phosphate together with 5’-phosphate termini. In the subsequent step in SP-BER, DNA polymerase β (pol β) with intrinsic 5’-deoxyribose phosphate lyase activity carries out both 5’-end cleaning and DNA repair synthesis with incorporation of a single nucleotide at the site of the base damage (9, 10). In case of LP-BER, ~2–6 nucleotides are incorporated by pol β or pol δ/ε, and the displaced 5’-flap is cleaved by flap endonuclease 1 (FEN1) prior to strand ligation (11, 12).

We and others have discovered a new family of human DNA glycosylases consisting of NEIL1 and NEIL2 (13–17) that are distinct in many ways from the previously identified oxidized base-specific glycosylases, 8-oxoguanine DNA glycosylase (OGG1) and endonuclease III homologue 1 (NTH1). A distinct, APE1-independent BER sub-pathway is responsible for NEIL1-initiated single nucleotide SP-BER (18) and involves a DNA repair complex comprising NEIL1, pol β, DNA ligase III α (lig III α), polynucleotide kinase, and x-ray repair cross-complementing 1 protein. Interestingly, NEILs stand apart from OGG1 and NTH1 in their ability to excise base lesions from bubble and single-stranded DNA (19), which might be transiently generated during DNA replication or transcription. The emerging concept of cellular interactions prompted us to search for significant protein partners of NEIL1 to further characterize its in vivo role. Earlier, we demonstrated stable interactions between NEIL1 and downstream BER proteins pol β and lig III α (18). In this study, we have identified stable association between NEIL1 and the Werner syndrome protein (WRN), a member of the RecQ family of DNA helicases. In addition to a 3’- to 5’-helicase activity, WRN also possesses a 3’- to 5’-exonuclease activity (20). WRN is mutated in the human autosomal recessive disease Werner syndrome (WS)
with a number of phenotypes, including premature aging, genome instability, and cancer predisposition (21, 22). WRN has been implicated in various DNA metabolic pathways, including replication, recombination, and repair (23, 24). A role for WRN in the repair of oxidative DNA damage is supported by its association with BER proteins as observed earlier, including APE1, pol β, FEN1, and poly(ADP-ribose) polymerase 1 (25–29). In addition, WS cells display elevated sensitivity to genotoxic agents, including methyl methanesulfonate (34), 4-nitroquinoline-1-oxide (30, 31), and ionizing radiation (32, 33).

Here, we provide evidence for a novel physical and functional interaction between WRN and NEIL1, whereby WRN stimulates the glycosylase activity of NEIL1. Oxidative stress enhances this association, and depletion of WRN leads to accumulation of oxidatively induced base lesions in cells. Taken together, our observations strongly suggest that WRN plays a significant role in oxidative DNA damage repair via an important association with NEIL1.

EXPERIMENTAL PROCEDURES

Cell Cultures and Knockdown Assays—The human colorectal carcinoma line HCT116 (expressing wild-type p53), a generous gift of Dr. B. Vogelstein (The Johns Hopkins University), was cultured in McCoy’s 5A modified medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml; Invitrogen). At 70% confluence, the cells were transfected and/or treated with glucose oxidase (GO, Roche Applied Science) at 100 ng/ml for 1 h, followed by washing with and incubation in fresh medium. This treatment did not affect cell viability, as judged by the rate of cell growth. The osteosarcoma cell line U-2 OS was cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, glutamine, penicillin, and streptomycin.

U-2 OS cells stably expressing a short hairpin RNA (Ambion) that is not homologous to any known human gene (WT) or a WRN short hairpin RNA (KD) were generated as described previously (34). Cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, glutamine, penicillin, streptomycin, and hygromycin B (0.2 mg/ml, Invitrogen).

For down-regulation of NEIL1, initially an ON-Target Plus SMART pool set of four (LU-008327-00-0005, Human NEIL1, NM_024608) siRNAs was purchased from Dharmacon and used according to the manufacturer’s protocol. This was followed by using each of the individual siRNAs (N1 to N4) to optimize NEIL1 depletion whereby the N3 (J-008327-06) siRNA resulted in maximum effect. Subsequently N3 was optimized at a dose of 80 nM to produce both single and double KD of NEIL1. Total RNA was isolated using an RNeasy mini kit (Qiagen) to monitor the level of NEIL1 expression by real-time qPCR analysis in the Protein Chemistry core facility (University of Texas Medical Branch, Galveston).

Proteins—Full-length NEIL1 and the C-terminal deletion fragments of NEIL1 (NEIL1Δ40 and NEIL1Δ101) were purified as described previously (14, 18). To examine the domain structure of NEIL1, we carried out limited proteolytic digestion of NEIL1 with endoproteinase Asp-N (Roche Applied Science). Two major non-overlapping domains consisting of N-terminal (residues 1–311) and C-terminal (residues 312–389) fragments were identified by N-terminal sequencing and mass spectroscopic analysis in the Protein Chemistry core facility (University of Texas Medical Branch, Galveston). The 78-amino acid C-terminal fragment of NEIL1 was purified as a GST-tagged polypeptide, from which the GST was cleaved by thrombin.

WRN wild-type or mutant fragments were cloned into Gateway pDEST 15 (Invitrogen) or pGEX-KG vectors and expressed and purified in Escherichia coli. Recombinant glutathione S-transferase (GST)-tagged proteins were purified as described previously (35). K-WRN (K577M) was expressed and purified using the Bac-N-Blue transfection kit (Invitrogen). High titer stocks were made, and the protein was expressed in Sf9 cells. The cells were lysed by freeze-thawing, and the protein was purified on a nickel-agarose column as described previously.

Co-immunoprecipitation—HCT116 cells were transfected separately with 1 μg of empty FLAG vector and FLAG-tagged expression constructs for NEIL1 (constructed in pCMV-C-FLAG, Sigma) and OGG1 (constructed in C-terminal pCMV-N-FLAG, Sigma) (36). 48 h post-transfection, cells were washed with phosphate-buffered saline (PBS) and lysed with cold lysis buffer containing 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, phosphatase inhibitors (Sigma), and a protease inhibitor mixture (Roche Applied Science). The cell extracts (2 mg/ml) were immunoprecipitated with anti-FLAG M2 antibody (Sigma), washed thoroughly with cold TBS (50 mM Tris-Cl, pH 7.5, 150 mM NaCl), and assayed for the presence of WRN in the complex by immunoblotting with antibody specific for the C terminus of WRN (Bethyl Laboratories). Oxidative stress on cells was imposed by GO treatment as described previously (36, 37), and co-immunoprecipitation analyses were carried out with mock and GO-treated cells.

GST-pulldown Assays—GST-pulldown assays were performed as described previously (26, 38). Briefly, glutathione- Sepharose beads (50 μl, 50% v/v), alone or bound to 1 ml of GST-tagged WRN polypeptides, were incubated for 2 h at 4 °C with 100 μg of HCT116 nuclear extract prepared as described previously (39) or purified recombinant NEIL1 (0.1 μg) in 250 μl of buffer (50 mM HEPES, pH 7.5, 100 mM KCl, 10% glycerol). The bound proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-NEIL1 antibody (Alpha Diagnostics, San Antonio, TX) and anti-GST antibody (GE Amersham Biosciences) for Western analysis.

Far Western Analysis—The proteins were separated by SDS-PAGE (10%), transferred to a nitrocellulose membrane, treated with 6 M guanidine-HCl, and then renatured with successive dilutions of guanidine-HCl in PBS, 1 mM dithiothreitol (40). After blocking with 5% nonfat dry milk in blocking buffer (PBS, 0.5% Tween 20), the membranes were incubated with the indicated protein in the blocking buffer for 3 h at 4 °C before immunoblotting analysis with corresponding antibodies.

Intrinsic Fluorescence Studies—The binding of WRN and NEIL1 was monitored by the decrease in intrinsic fluorescence of WRN HcRQC tryptophan residues (λex = 295 nm, λem = 300 – 450 nm) upon titration with NEIL1 peptide78. All binding experiments were performed in buffer A (20 mM Tris, 1 mM EDTA, 2 mM MgCl2, and 150 mM KCl, pH 7.5) at 25 °C for an
incubation time of 5 min. The fluorescence was measured in a LS50 spectrophotometer (PerkinElmer Life Sciences) in a QS cell of light path 1 cm. HlcRQC (0.25 μM) was titrated with increasing concentrations (0 to 0.25 μM) of NEIL1 peptide78. Spectra were corrected for buffer scattering and sample dilution. The intensity of Raman scattering was recorded (NEIL1p78 contains no tryptophan residues) for the titration by adding NEIL1 peptide78 to the HlcRQC solution, where addition of NEIL1p78 to buffer in a similar manner was served as blank. Each fluorescence spectrum represents an average of five dilutions. The intensity of Raman scattering was recorded (Spectra were corrected for buffer scattering and sample dilution). The bound proteins were separated by SDS-PAGE and probed with anti-NEIL1 and anti-GST antibody.

**DNA Strand Incision Assay for NEIL1**—NEIL1 substrates were used. UV irradiation of DNA containing 3′- or 5′-phosphates was achieved with 365-nm light (120–150 J/m²) using a transilluminator. The damaged bases were determined by plasmid isolation, end-labeling with [γ-32P]ATP, and electrophoresis on a 15% polyacrylamide gel. The products were separated by SDS-PAGE and probed with anti-NEIL1 antibody. The damage-containing oligonucleotides, named B11US and B12US (Invitrogen), respectively, containing FapyG at residue 13 (indicated by G) and 36-mer oligonucleotides, with FapyG at residue 13 (indicated by G) were prepared as previously described (43). The damage-containing oligonucleotides, named B11US and B12US, were labeled with [γ-32P]ATP and polynucleotide kinase before annealing to the complementary oligonucleotides, B11DS and B12 US (Invitrogen), respectively, containing a C opposite the lesion. DNA strand cleavage at the site of the lesion due to the intrinsic AP-lyase activity of NEIL1 after base excision was assayed by incubation of 32P-labeled X or Y containing substrates with NEIL1 (20 fmol) alone or together with WRN (20–80 fmol) or its fragments as indicated in a buffer containing (40 mM Tris, pH 8, 4 mM MgCl₂, 5 mM dithiothreitol, and 0.1 mg/ml BSA) at 37 °C for 15 min. After stopping the reaction with 70% formamide/30 mM NaOH, the products were separated on a 12% native polyacrylamide gel and visualized using a Phosphorlmager (Amersham Biosciences).

**Exonuclease Reactions** were performed essentially as described previously (29). Briefly, oligonucleotide Bub19T was 5′-end-labeled and annealed to Bub19B. Reactions (10 μl) were performed in helicase reaction buffer (see above), DNA substrate (1 nM), WRN, and NEIL1 as indicated. Samples were incubated for 15 min at 37 °C, and the reaction was terminated by the addition of formamide stop dye (80% formamide, 0.5× TBE, 0.1% bromphenol blue, and 0.05% xylene cyanol). The products were heat-denatured at 90 °C for 5 min, separated on a 14% denaturing polyacrylamide gel, and analyzed as before.
For enzyme kinetics studies, the oligonucleotides (3.2–125 nm) were incubated with NEIL1 or NEIL1 plus WRN for 4 min at 37 °C. The linear reaction rate, $K_m$, and $k_{cat}$ were calculated by linear regression analysis using SigmaPlot.

Quantitation of 8-oxo-G, FapyG, and FapyA—8-Oxo-G was measured as its nucleoside 8-oxo-dG in cellular DNA by liquid chromatography/mass spectrometry with isotope dilution technique using 8-oxo-dG-15N5 as an internal standard as described previously (44). FapyG and FapyA were measured by gas chromatography/mass spectrometry as described previously (45). Statistical significance was determined by paired t test and analysis of variance.

**Immunostaining and Confocal Imaging**—5 × 10⁴ cells per dish were grown to 50% confluency on glass coverslips (25 mm) in 5% CO₂ and at 37 °C. After treatment with and without GO, the cells were washed with PBS, dried, and fixed in acetone-methanol 1:1 (v/v). After exposure to heterologous pre-immune serum (0.1 μg in PBS containing 0.05% Tween 20 and 0.5% BSA; PBS-T) for 30 min, the cells were incubated with primary antibody (rabbit polyclonal anti-NEIL1 or mouse monoclonal anti-WRN), and counterstained with fluorescein-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min at 37 °C and washed in PBS-T. The cell nuclei were stained for 15 min with 4,6-diamidino-2-phenylindole dihydrochloride, 10 ng/ml, and mounted in anti-fade medium (Dako Inc., Carpinteria, CA) on microscope slides. Confocal microscopy was performed on a Zeiss LSM510 META system with the 488 nm line of the argon laser for excitation of fluorescein isothiocyanate and helium-neon 543 nm line excitation of rhodamine, combined with appropriate dichroic mirrors, and emission band filters to discriminate between green and red fluorescence. Images were captured at a magnification of ×60. Co-localization was visualized by superimposition of green and red images using MetaMorph software version 4.6r9 (Universal Imaging Corp.). The co-localization coefficient shows extent of superimposition in the green channel with respect to the red channel. Briefly, the regions of interest were drawn to include the structures after the individual channels were thresholded. Scatter plots were generated, and coefficients of co-localization were calculated (46, 47).
Functional Interaction between NEIL1 and WRN

RESULTS

Identification of WRN as a NEIL1 Interacting Protein—NEIL1 has previously been shown to stably interact with several BER proteins (18). To further elucidate the cellular role played by this enzyme in oxidized base repair we decided to identify other possible NEIL1 interacting partners by co-immunoprecipitation analysis. For this HCT116 cells were transfected with NEIL1-FLAG, OGG1-FLAG, or empty FLAG expression plasmids, and whole cell extracts were immunoprecipitated with anti-FLAG antibody beads (Sigma). WRN could be identified as an interacting protein in the NEIL1-FLAG IP but not in the vector IP or OGG1 IP (Fig. 1A) when probed with anti-WRN antibody. Equal amounts of lysates used for pulldown assays are shown as input. The absence of WRN in an OGG1 IP provided an appropriate negative control, as well as evidence for the specificity of the WRN/NEIL1 association. These results indicate the existence of WRN in a complex with NEIL1 in vivo.

Direct Association between NEIL1 and WRN: Mapping of WRN Interacting Domain—To test for physical interaction between NEIL1 and WRN, we performed affinity pulldown experiments with various recombinant WRN fragments expressed as GST-fusion polypeptides and nuclear extracts from HCT116 cells. As shown in Fig. 1B, NEIL1 co-precipitated with GST-WRN949–1432 (lane 3), GST-WRN949–1092 (lane 2), and to a smaller extent with GST-WRN1072–1432 (lane 1) by comparison with the input (lane 6). Purified GST (lane 5) or GST-WRN239–499 (lane 4) did not co-precipitate NEIL1. There was some cross-reactivity of the anti-NEIL1 antibody to bacterial proteins migrating at lower molecular weight. To rule out the possibility that other proteins in the extracts mediated the interaction between WRN and NEIL1, a GST-pulldown assay was also performed with recombinant NEIL1 protein. As before, we observed that the N-terminal recombinant GST-WRN fragments failed to pull down recombinant NEIL1, whereas C-terminal fragments were bound to NEIL1 to varying degrees (Fig. 1C), providing strong support for a direct interaction between NEIL1 and WRN. Thus the RQC region (WRN883–1075 by sequence homology), which overlaps with most of the GST-WRN fragment, 949–1092, was mapped as the WRN interacting domain that binds to NEIL1.

Mapping of the Interacting Region of NEIL1 with WRN—We next mapped the NEIL1 interacting domain for WRN by far Western analysis using purified full-length and deletion constructs of NEIL1. NEIL1 has a common domain near the C terminus for interaction with BER proteins, pol β and lig III α (18). The interaction domain of NEIL1 spanning residues 288 to 349 could be mapped using the C terminally truncated NEIL1 polypeptides (CA40 and CA101) lacking the C-terminal aa residues 40 and 101, respectively. We observed stable interaction of WRN with CA40, CA101, and full-length NEIL1 (Fig. 2A). OGG1, another oxidized base-specific DNA glycosylase, did not directly interact with WRN, and thus served as a negative control.

Limited proteolytic digestion indicated the presence of two major domains in NEIL1; the N-terminal domain spanning residues 1–311 and the C-terminal domain with residues 312–389 (Fig. 2B). The C-terminal NEIL1 fragment comprising the 78 aa was then used as a probe, and it revealed binding to full-length as well as to the HcRQC domain of WRN but not to the Hc domain of WRN (Fig. 2C). Taken together, these results confirm our previous studies and indicate that WRN interacts via its RQC domain with NEIL1.

Stability of the WRN Interaction with NEIL1—The binding of NEIL1 to WRN was analyzed from dose-dependent quenching of intrinsic tryptophan fluorescence of the HcRQC domain of WRN by the NEIL1 probe of 78 aa, which lacks Trp or Tyr. The emission maximum for WRN HcRQC was observed at 345 nm with excitation at 295 nm (Fig. 3A). As expected, the 78-aa peptide has negligible fluorescence (Fig. 3A). Non-linear regression analysis using a ligand-receptor binding formula ("Experimental Procedures") showed an apparent KD of 60 nM (Fig. 3B) for the binding of WRN interacting domain to NEIL1 polypeptide, indicating a very high affinity. A comparable KD was observed for the binding of full-length WRN to the NEIL1 polypeptide (data not shown).

Inhibition of WRN Activity by NEIL—The stable interaction between NEIL1 and WRN raised the possibility of their ability to modulate each other’s activity. We observed that NEIL1 inhibited WRN helicase activity in a concentration-dependent manner, with both bubble and forked duplex DNA substrates (Fig. 4A). The helicase activity of WRN was inhibited ~40% by 2-fold and nearly 80–90% by 10-fold molar excess of NEIL1.
Competitive binding experiments revealed that the observed inhibition was a consequence of non-cooperative binding. When WRN was added in excess, NEIL1 activity was found to be inhibited in a non-cooperative manner, with an IC50 value of 2.5 nM (lanes 7 and 8, 12.5 nM) (lanes 2, 9, 13, and 19). Lanes 10 and 20, heat-denatured substrate. Reaction products were run on a 12% native gel and visualized using a PhosphorImager.
2-fold in the presence of WRN with the 5-OHU.B11 substrate, along with ~6-fold increase in $k_{cat}$. These results indicate that WRN stimulates NEIL1 activity both by enhancing its affinity for the damage-containing substrate (supplemental Fig. S1) and increasing the $V_{max}$ or reaction rate. We confirmed that WRN stimulation of NEIL1 is independent of the lesion type by measuring NEIL1 activity with bubble substrates containing 8-oxo-G and FapyG. Although less efficient than 5-OHU cleavage (14), NEIL1 incision of the 8-oxo-G oligonucleotide, was also stimulated significantly by WRN (Fig. 5B, lanes 7 and 8). In addition FapyG was efficiently cleaved by NEIL1, and the cleavage activity was enhanced by the presence of WRN (Fig. 5B, lanes 3 and 4). In our present studies we focused on the activity of NEIL1 on FapyG, 5-OHU, and 8-oxo-G lesions present in oligonucleotide substrates, because the sensitivity of the glycosylase assay with irradiated natural DNA containing low levels of multiple lesions is much less than with specific lesion-containing oligonucleotides (14).

**NEIL1 Stimulation Is Independent of WRN Helicase Activity**—WRN stimulation of NEIL1 activity was ATP-independent, suggesting that the helicase activity is dispensable for NEIL1 stimulation. Our observation that the helicase-deficient WRN-K577M mutant (48, 49) stimulated NEIL1 supported this possibility (supplemental Fig. S2). Furthermore, the recombinant helicase domain (Hlc) by itself did not affect NEIL1 activity (Fig. 5C, lanes 7 and 8), whereas recombinant HlcRQC stimulated NEIL1 3-fold (Fig. 5C, lanes 5 and 6). Furthermore, the GST-RQC domain alone was capable of enhancing NEIL1 activity (Fig. 5C, lanes 3 and 4). Taken together, these observations indicate that the RQC domain of WRN is necessary and sufficient for NEIL1 stimulation.

**Measurement of Oxidative Damage in NEIL1 and WRN Knockdown Cells**—Reactive oxygen species (ROS) generated as by-products of cellular respiration produce numerous base

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**TABLE 2**

| Reaction        | $K_m$ (nM) | $k_{cat}$ (10$^{-2}$/min) |
|-----------------|------------|---------------------------|
| NEIL1 only      | 13.2 ± 1.4 | 9.3 ± 0.3                 |
| NEIL1 plus WRN  | 6.8 ± 0.8  | 57.5 ± 1.6                |
modifications, including 8-oxo-G, 5-OHU, thymine glycol, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG), and 4,6-diamino-5-formamidopyrimidine (FapyA) (44). To probe the in vivo role of WRN, we investigated the effect of its down-regulation on the level of oxidatively modified bases in the cell genome. We quantitated several prototypical oxidized bases, namely 8-oxo-G, FapyG, and FapyA, the latter being well characterized substrates of NEIL1, in WT and WRN knockdown (KD) cells by using liquid chromatography/mass spectrometry and gas chromatography/mass spectrometry analyses, respectively. We observed ~50% higher levels of 8-oxo-G in WRN KD cells compared with WT cells (Fig. 6B, upper left panel) and also some increase in FapyG levels (Fig. 6B, upper right panel). We went on to probe the oxidative repair pathway in which WRN seems to play an important role. For this we knocked down NEIL1 in WT as well as WRN KD cells using siRNA (Dharmacon) as described under “Experimental Procedures,” whereby 80% of NEIL1 expression was down-regulated (Fig. 6A). As expected, FapyG level was found to be significantly elevated in NEIL1 single KD cells (Fig. 6B, bottom panel). Interestingly, a double knockdown of WRN with NEIL1 did not reveal any additional damage accrual (Fig. 6B, bottom panel) suggesting that WRN lies in the same repair pathway as NEIL1. A somewhat similar result was obtained when FapyA was measured in NEIL1 and WRN KD cells (Fig. 6C). No difference in ROS production was observed as a result of down-regulation of NEIL1 or WRN (data not shown). This confirms that the difference in the lesion level did not result from increased ROS production in the knockdown cells.

WRN Association with NEIL1 Is Enhanced in Vivo by Oxidative Stress—We next investigated whether oxidative stress affects the in vivo association of WRN with NEIL1. HCT116 cells stably expressing
NEIL1-FLAG were mock treated or treated with a subtoxic dose of GO (100 ng/µl), which results in ~2.5-fold increase in the ROS level (37). Co-immunoprecipitation analyses revealed greater pull down of WRN by NEIL1-FLAG as a result of GO treatment (Fig. 7A). Microscopic analysis of intracellular distribution of NEIL1 and WRN in mock and oxidatively stressed cells was then performed. WRN was previously shown to localize primarily in the nucleoli of human cells (50) and to relocalize in the nucleoplasm in response to genotoxic agents. In the case of NEIL1, exposure of cells to ROS results in its accumulation in the nucleus. Our results showed that in mock treated HCT116 cells, NEIL1 was distributed both in cytoplasm and nuclei, whereas WRN was localized to the nucleolus, with a modest level of co-localization with NEIL1, the co-localization coefficient being 0.11 ± 0.9 from three independent experiments (data not shown). In oxidatively stressed cells, NEIL1 and WRN were both accumulated in the nucleoplasm, as speckles/foci; superimposition of NEIL1 and WRN images showed a much stronger co-localization (co-localization coefficient = 0.89 ± 0.6) (Fig. 7B). These results provide significant support for the early recruitment of WRN along with NEIL1 particularly in response to oxidative stress-induced DNA damage.

**DISCUSSION**

Oxidative DNA base damage is typically repaired via the BER pathway. We had shown earlier that the mammalian DNA glycosylase, NEIL1, an ortholog of *E. coli* Nei/Fpg (14–16, 51) preferably excises oxidative damage from single-stranded and bubble DNA substrates (19). Its substrates include a wide range of oxidatively damaged bases, including 5-OHU, thymine glycol, 8-oxo-G, FapyG, and FapyA (14, 51). Significant down-regulation of NEIL1 resulted in increased sensitization of mouse embryonic fibroblasts to ionizing radiation (52), in comparison to OGG1- and NTH1-null cells (17, 53). We also found up-regulation of hNEIL1 by oxidative stress (37), which underscored its role in protecting cells from the genotoxic effects of ROS. A recent report indicates that NEIL1 null (Neil1−/−) mice accumulate mitochondrial DNA damage and develop dyslipidemia, obesity, diabetes, and fatty liver disease (54). The pathogenesis of this metabolic syndrome could be partly attributed to ROS exposure (54, 55). These data strongly suggest that NEIL1 is not simply a backup glycosylase for OGG1 and NTH1 but has a unique and significant function in vivo.

In this report, we have documented physical and functional interaction between NEIL1 and WRN, this being the first report of the association of WRN with a DNA glycosylase of the BER pathway. The lack of association between WRN and OGG1, another glycosylase, underscores the specificity of WRN and NEIL1 binary interaction. We have shown here that the C-terminal RQC domain of WRN, the major DNA and protein binding domain (56), is sufficient to mediate the interaction with NEIL1 and to stimulate oxidative base incision by NEIL1 in an ATP-independent manner. Kinetic analyses of the NEIL1 cleavage reaction for damage-containing substrates further demonstrate that WRN increases the catalytic specificity of NEIL1.
NEIL1 by affecting the reaction rate at subsaturating DNA concentrations. Biochemical studies of ours and others have revealed that the RQC domain of WRN mediates functional interactions with several members of the recombination and repair pathways, including other BER proteins like pol β, FEN1, and PARP-1 (57). In the case of NEIL1, the interaction module lies just downstream of its zinc-less DNA binding motif harboring the conserved Arg-277 (58). WRN binds to NEIL1 with very high affinity ($K_D = 60$ nM) at nearly equimolar stoichiometry.

We have previously shown NEIL1 to be present at ~30,000 molecules/cell (59), whereas others have reported the concentrations of WRN to be an average of 60,000 molecules/cell (60). Thus, the number of WRN molecules/cell is ~2-fold than that of NEIL1, supporting that the activation of NEIL1 by WRN observed in our studies is likely to be physiologically significant.

Probing for the cellular roles of NEIL1 and WRN in response to oxidative damage repair provided some interesting insights. WRN deficiency alone showed some increase in genomic Fapy levels (Fig. 6, B and C), which might be accounted for by a lack of stimulation of Fapy removal by the preferred glycosylase NEIL1 or by dysfunction of a parallel repair pathway involving WRN. The level of Fapys was elevated as predicted in NEIL1-depleted cells (Fig. 6, B and C). The lack of additive effect of depleting both WRN and NEIL1 could be explained by the model that WRN is part of the NEIL1 repair complex. Depletion of WRN in cells also result in some accrual of the lesion 8-oxo-G (Fig. 6B). OGG1 is the preferred glycosylase for 8-oxo-G, although this lesion is also excised by NEIL1 to some degree (18, 19). This excision activity is enhanced in presence of WRN. Our previous work showed that NEIL1 influences OGG1 turnover and improves the latter’s activity on 8-oxo-G removal (18, 59). In the absence of any direct collaboration between OGG1 and WRN, reduced levels of WRN in the cell indirectly imply a lack of stimulation of more efficient 8-oxo-G removal by NEIL1.

Finally, the cooperation between NEIL1 and WRN during DNA damage response signaling gained support from our observation that oxidative stress enhanced the physical association of these two proteins (Fig. 7A). One likely mechanism is enhanced affinity due to their covalent modifications (e.g. phosphorylation, acetylation, monoubiquitylation, etc). Previous reports indicate that WRN plays a role in the cellular response to DNA damage, and its activity is possibly modulated by DNA damage-induced acetylation of WRN and/or its interacting partners (61). A similar oxidative challenge of cells resulted in greater spatial overlap or co-localization of NEIL1 and WRN as evidenced from their foci formation in the stressed cell nuclei (Fig. 7B).

We had previously suggested a model whereby WRN acts on a late BER intermediate, formed after the action of APE1 (25). In the present modified model (Fig. 8), we propose recruitment of WRN at a very early stage of damage recognition. The first step of BER involves a lesion-specific component, NEIL1, a bifunctional DNA glycosylase/AP lyase that recognizes the oxidized base and recruits several repair-linked proteins including WRN. WRN remains bound to NEIL1 at the damaged site and increases damage removal by NEIL1. NEIL1 itself remains associated with the damaged DNA after it has performed its AP lyase activity and might play a role in preventing unwanted unwinding by WRN at the nick. Because NEIL1 is involved in an APE1-independent sub-pathway (18), polynucleotide kinase would be the next downstream protein, which we could detect in the NEIL-WRN immunocomplex. Subsequently, pol β and lig III α come into play and at this stage the helicase activity of WRN might be recruited to unwind the single-strand breaks and stimulate strand displacement DNA synthesis of pol β (27), completing repair by the short-patch (SP-BER) pathway. WRN also may play a critical role by interacting with and stimulating FEN1 activity in long-patch or LP-BER (26). Thus our model (Fig. 8) briefly illustrates involvement of WRN in the early as well as late steps of BER via several parallel pathways that utilizes a hand-off mechanism for sequential recruitment of multiple repair factors. While this report was being prepared there was a parallel observation of NEIL1 interaction with the 9-1-1 complex (66). It will be interesting in the future to probe the mechanism by which NEIL1 or WRN switches their partners in response to signal from oxidative/genotoxic stress within the cell.

Das, A., and Bohr, V. A., unpublished observation.

FIGURE 8. A model of the proposed role of WRN in BER. Model depicts early recruitment of WRN in the damage-sensing step in association with NEIL1 BER complex. In addition, involvement of WRN is shown in parallel SP-BER pathways in association with APE1 and in LP-BER involving FEN1. Details are given under "Discussion."
The distinct feature of NEIL1 compared with other glycosylases in utilizing bubble and forked substrates (19) implicates NEIL1 in the repair of DNA in actively transcribed regions of the genome. Thus, the role of the WRN-NEIL1 complex in the repair of oxidative damage may be associated with particular regions of the genome such as open chromatin regions. It is tempting to speculate as to the role of WRN in BER especially under certain metabolic conditions (such as during replication or transcription), and this specificity can account for the lack of prominent general BER defects at the global level (global genome repair) in WS cell extracts.

Previous reports show abnormal sensitivity to the ROS-producing agent, hydrogen peroxide, in WS cells and in WRN-knockdown MRC-5 normal fibroblasts using the Comet assay (62). Recently, it was shown that growth in physiological (3%) oxygen or in the presence of an antioxidant prevented the formation of the DNA damage foci in WRN-depleted cells, whereas acute oxidative stress led to inefficient repair of the lesions (63). These results strongly suggest that DNA lesions arising due to ROS or other oxidative damage cannot be fully repaired by WRN-independent mechanisms. Again, redox abnormalities in WS cells and WS patients suggest that a deficiency in WRN may be a basis for the in vivo pro-oxidant state (64), which causes oxidative damage to biomolecules and multiple oxidative stress-related alterations. This report is the first demonstration of a direct involvement of WRN in the damage-sensing pathway that is initiated by the oxidative base-specific enzyme, NEIL1. Earlier studies suggested that NEIL1 activation by ROS is part of a coordinated cellular response to the additional load of oxidative DNA damage both in the nucleus and mitochondria. How the functional association of NEIL1 with WRN modulates this response in the context of global genome repair and also in aging pathologies warrants further investigation.

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