Interaction of the Human DNA Glycosylase NEIL1 with Proliferating Cell Nuclear Antigen

THE POTENTIAL FOR REPLICATION-ASSOCIATED REPAIR OF OXIDIZED BASES IN MAMMalian GENOMES

NEIL1 and NEIL2 compose a family of DNA glycosylases that is distinct from that of the other two DNA glycosylases, OGG1 and NTH1, all of which are involved in repair of oxidized bases in mammalian genomes. That the NEIL proteins, unlike OGG1 and NTH1, are able to excise base lesions from single-stranded DNA regions suggests their preferential involvement in repair during replication and/or transcription. Previous studies showing S phase-specific activation of NEIL1, but not NEIL2, suggested NEIL1 involvement in the repair of replicating DNA. Here, we show that human NEIL1 stably interacts both in vivo and in vitro with proliferating cell nuclear antigen (PCNA), the sliding clamp for DNA replication. PCNA stimulates NEIL1 activity in excising the oxidized base 5-hydroxycytosine from single-stranded DNA sequences including fork structures. PCNA enhances NEIL1 loading on the substrate. In contrast, although present in the NEIL2 immunocomplex, PCNA does not stimulate NEIL2. NEIL1 interacts with PCNA via a domain that is located in a region near the C terminus, dispensable for base excision activity. The interacting sequence in NEIL1, which lacks the canonical PCNA-binding motif, includes a sequence conserved in DNA polymerase δ and implicated in its PCNA binding. Mammalian two-hybrid analysis confirmed PCNA interaction with NEIL1. The G127A mutation in PCNA reduces its stimulatory activity, suggesting that the interdomain connector loop, a common binding interface of PCNA, is involved in NEIL1 binding. These results strongly support in vivo function of NEIL1 in preferential repair of oxidized bases in DNA prior to replication.

Reactive oxygen species, ubiquitous genotoxic agents, are continuously generated in vivo as by-products of respiration and are also produced as a result of metabolism of toxic agents or induced during inflammatory responses (1–3). Reactive oxygen species have been implicated in the etiology of a wide variety of diseases ranging from arthritis to cancer and also in aging (4). Reactive oxygen species induce a plethora of oxidatively damaged bases, abasic (apurinic/apyrimidinic (AP))5 sites, and strand breaks in the genome that, if left unrepaird, could lead to mutagenesis, apoptosis, senescence, and sporadic cancer (4). Such damage, with the exception of double strand breaks, is repaired primarily via the DNA base excision repair (BER) pathway (5).

The BER pathway, first delineated in Escherichia coli, is initiated with excision of the damaged base by a DNA glycosylase. Oxidized base-specific glycosylases, all with intrinsic AP lyase activity, then cleave the DNA strand at the resulting AP site, generating a 3’-terminal deoxyribose phosphate or 3’-phosphate (6). After removal of this 3’-blocking group, the single nucleotide gap is filled in by a DNA polymerase, using the undamaged complementary strand as a template, and DNA ligase finally seals the nick to restore duplex DNA (5, 7). The oxidized base-specific DNA glycosylases have broad substrate specificity commensurate with the large number of such lesions and are conserved in organisms ranging from bacteria to mammals (6, 8). Recent reports indicate that the BER process is more complex and involves many additional proteins, particularly for repair of oxidative lesions and AP sites in mammalian cells (5, 9–11).

Mammalian cells have two distinct BER subpathways defined by the repair patch size. As described above, a single nucleotide gap is generated by the AP lyase activity of oxidized base-specific DNA glycosylases or after endonucleolytic cleavage of an AP site and removal of the 5’-blocking group, the single nucleotide gap is filled in by a DNA polymerase, using the undamaged complementary strand as a template, and DNA ligase finally seals the nick to restore duplex DNA (5, 7). The oxidized base-specific DNA glycosylases have broad substrate specificity commensurate with the large number of such lesions and are conserved in organisms ranging from bacteria to mammals (6, 8). Recent reports indicate that the BER process is more complex and involves many additional proteins, particularly for repair of oxidative lesions and AP sites in mammalian cells (5, 9–11).

The abbreviations used are: AP, apurinic/apyrimidinic; BER, base excision repair; pDNA, polymerase; SN-BER, single nucleotide base excision repair; APE, apurinic/apyrimidinic endonuclease; 5-OHU, 5-hydroxycytosine; 5-OHU, 5-hydroxycytosine; PCNA, proliferating cell nuclear antigen; WT, wild-type; GST, glutathione S-transferase; BSA, bovine serum albumin; EMSA, electrophoretic mobility shift assay; SPR, surface plasmon resonance; RFC, replication factor C; LP-BER, long patch base excision repair.
This process is defined as single nucleotide base excision repair (SN-BER). In an alternative pathway that was delineated with reconstituted systems for repair of synthetic (tetrahydrofuran) or reduced AP sites, the 5'-flap endonuclease (FEN1) with 5'-exonuclease activity removes the 5'-sugar phosphate, the cleavage product of AP, or its analogs along with additional (two to six) deoxynucleotides from the 5' terminus. Repair synthesis is carried out by a replicative polymerase such as pol δ/ε, and repair is completed by DNA ligase I (13, 14).

One unexplored issue in BER is the need for preferential repair of mutagenic oxidized base lesions in functional regions of the genome. Most adult tissues contain nondividing, terminally differentiated cells in which DNA base lesions will not induce mutations. Furthermore, because only a small fraction of the mammalian genome contains transcription units, the repair of mutagenic lesions in untranscribed sequences should not be as urgent as that in transcribed sequences. Additionally, the damage in the nontranscribed strand may not affect transcription. However, repair of mutagenic oxidized bases in the nontranscribed strands of actively dividing cells, e.g. during development and in regenerative tissues, should be as critical as that in the transcribed strands. In such cases, mutations would be fixed due to damage in either strand. This is particularly important for oxidatively damaged bases, which, unlike bulky adducts, do not usually block replication or transcription (15–18). We (6) and others (5) have postulated a model of replication-associated repair in which the repair enzyme scans the genome to repair damaged bases in the template strand before replication, to prevent mutations. A likely prerequisite of replication-associated repair is the cross-talk between DNA replication proteins and BER enzymes.

Three oxidatively damaged base-specific DNA glycosylases (Nth, Fpg, and Nei) have been characterized in *E. coli* and, based on their tertiary structures, active-site characteristics, and AP lyase activity, are divided into two groups. Nth is the prototype of one, and Fpg/Nei of the other (19). The two previously characterized oxidized base-specific mammalian DNA glycosylases, OGG1 and NTH1, belong to the Nth family (5, 20–23). We characterized two additional human DNA glycosylases (subsequently confirmed by others) that belong to the Fpg/Nei family and named them NEIL1 and NEIL2 (23–28). We have recently shown that NEIL-initiated BER does not involve apurinic/apyrimidinic endonuclease (APE), unlike the situation in *E. coli*, but requires polynucleotide kinase, which is present in mammalian cells but absent in *E. coli* (29). NEIL proteins carry out SN-BER mediated by polynucleotide kinase, pol β, and DNA ligase IIIα with XRCC1 (29, 30). Stable interaction of NEIL proteins with the downstream repair proteins pol β, XRCC1, and DNA ligase IIIα led us to propose that these DNA glycosylases, as the first enzymes in the repair process, determine the specific subpathway for repair (29, 30).

NEIL1 and NEIL2 have dissimilar sequences, but share several common characteristics, including overlapping substrate range and preference for the substrate lesion in single-stranded DNA sequences like those present in a transcription bubble or a replication fork (31). A common damaged base substrate of these enzymes as well as of NTH1 is 5-hydroxyuracil (5-OHU), an oxidation product of cytosine. In contrast to the NEIL proteins, OGG1 and NTH1, like most other glycosylases, are inactive with single-stranded DNA substrates (31). We thus hypothesized that NEIL1 and NEIL2, unlike OGG1 or NTH1, are involved in repair associated with replication and/or transcription by excising oxidized bases from transient bubble and fork intermediates (31). However, the NEIL proteins have distinct cell cycle regulation. Although NEIL2 expression is cell cycle-independent, we observed that NEIL1 expression is strongly dependent on the S phase (24, 25). That led us to test the hypothesis that NEIL1 and not NEIL2 is preferentially responsible for replication-associated repair. In support of this, we now describe here the stable interaction of NEIL1 with the proliferating cell nuclear antigen (PCNA) sliding clamp, which is essential in DNA replication by acting as a scaffold for proteins present in the replication complex.

**Experimental Procedures**

**Cell Culture**—The human embryonic kidney cell line HEK293 was maintained and grown in Dulbecco’s modified Eagle’s medium at 37 °C and 5% CO₂. The human colorectal tumor line HCT116 (with wild-type p53) was grown in McCoy’s 5A medium at 37 °C and 5% CO₂. All media contained 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. All reagents were purchased from Invitrogen.

**Oligonucleotide Substrates**—A 51-mer oligonucleotide containing 5-OHU at position 26 from the 5’-end was purchased from Midland Co. The undamaged 51-mer control oligonucleotide had Gua opposite the lesion for generating duplex or contained noncomplementary sequences for producing bubble and fork structures as shown in Table 1. For optimal annealing, an equimolar mixture of lesion-containing and complementary strands was heated to 94 °C for 2 min in phosphate-buffered saline and then slowly cooled to room temperature. The 5-OHU-containing oligonucleotide was ³²P-labeled at the 5’ terminus with [γ-³²P]ATP using polynucleotide kinase prior to annealing when necessary.

**Plasmids**—Mammalian expression plasmids for FLAG-tagged NEIL1 and NEIL2 were described previously (30, 32). Construction of bacterial expression plasmids for the wild-type (WT) and truncated forms of NEIL1 has also been described (29). To generate bacterial expression plasmids for the production of N-terminally glutathione S-transferase (GST)-fused NEIL1 C-terminal domains, the coding sequences for NEIL1 (289–389) and NEIL1-(289–349) were amplified from NEIL1 cDNA by PCR with the addition of BamHI and EcoRI restriction sites. The PCR products were then subcloned into the pGEX-2T vector at the BamHI and EcoRI sites in-frame with GST. To generate plasmids for the mammalian two-hybrid system (Stratagene), NEIL1 and PCNA were PCR-amplified and subcloned using BamHI and XbaI sites into pCMV-BD and pCMV-AD, respectively. Site-directed mutants were generated using the QuikChange® site-directed mutagenesis kit (Stratagene). All recombinant plasmid sequences were confirmed by DNA sequencing.

**Expression and Purification of Recombinant Proteins**—Recombinant WT NEIL1, WT NEIL2, and truncated NEIL1...
polypeptides were purified to homogeneity from *E. coli* as described previously (24, 25, 29, 30). His-tagged PCNA was purified by affinity chromatography on a Ni²⁺ column, followed by chromatography for final purification on a HiTrap-SP column (GE Healthcare). Recombinant WT PCNA was expressed in *E. coli* and purified according to Matsumoto et al. (13). GST-fused NEIL1-(289–349) and NEIL1-(289-389) were expressed in *E. coli*, purified from the cell extract by glutathione–Sepharose affinity chromatography (GE Healthcare), and then eluted using 20 mM reduced glutathione. The GST fusion peptides were dialyzed and subjected to another step of purification by cation exchange chromatography.

**Assay of DNA Glycosylase Activity**—The DNA glycosylase activity of NEIL proteins was quantitated on the basis of strand incision at the 5-OHU site after its excision from 5-OHU-32P-labeled DNA oligonucleotide substrates. After incubation of the DNA (25 nM) with NEIL1 or NEIL2 in a 10-μl reaction mixture containing 40 mM HEPES-KOH (pH 7.5), 50 mM KCl, 1 mM MgCl₂, and an appropriate amount of bovine serum albumin (BSA) to maintain a constant protein level in the presence or absence of PCNA at 37 °C for the indicated times, the reaction was stopped with 70% formamide and 30 mM NaOH. The alkali in the stop buffer cleaved any uncleaved AP sites that escaped NEIL AP lyase activity. The intact and cleaved oligonucleotides were then separated by denaturing gel electrophoresis on 20% polyacrylamide containing 7 M urea, 90 mM Tris borate (pH 8.3), and 2 mM EDTA, and the radioactivity in the DNA bands was quantitated in a PhosphorImager using ImageQuant software (GE Healthcare).

For single turnover enzyme kinetics, we incubated 5-OHU bubble oligonucleotide substrate (2 nM) with excess NEIL1 (20 nM) alone or in the presence of PCNA (0.5 μM) at 37 °C in buffer containing 40 mM HEPES-KOH (pH 7.5), 50 mM KCl, and 1 mM MgCl₂. The reaction was stopped at the designated times, and cleaved products were quantitated as described above.

**Immunoblotting**—Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes, which were then blocked in Tris-buffered saline (50 mM Tris-HCl (pH 7.5) and 150 mM NaCl) with 0.05% Tween 20 and 5% nonfat dried milk and incubated with the specified antibodies. The membranes were washed three times, followed by incubation with the appropriate anti-mouse, anti-rabbit, or anti-goat secondary antibodies linked to horseradish peroxidase (GE Healthcare) when necessary. The immunocomplexes were detected by enhanced chemiluminescence (GE Healthcare). The antibodies used included mouse anti-PCNA (clone PC10), rabbit polyclonal anti-NEIL1 (24), horseradish peroxidase-conjugated anti-FLAG M2 (Sigma), horseradish peroxidase-conjugated anti-His tag (Santa Cruz Biotechnology, Inc.), and goat anti-GST (GE Healthcare).

**Co-immunoprecipitation Assay**—HEK293 cells were transfected with empty FLAG, NEIL1-FLAG, or NEIL2-FLAG plasmid. The cells were collected 48 h after transfection and lysed by sonication (15% output, 10 s) in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM NaF, 1 mM sodium orthovanadate, and β-mercaptoethanol plus protease inhibitors. In several experiments, the cell lysates were digested with 500 units/ml DNase I (Ambion) at 37 °C for 30 min and cleared by centrifugation. The lysates were then immunoprecipitated for 3 h at 4 °C with anti-FLAG antibody M2 cross-linked to agarose beads (Sigma). The beads were collected by centrifugation and washed three times with cold Tris-buffered saline, and the FLAG immunocomplex was eluted from the beads by adding SDS loading buffer. The immunocomplex was separated by SDS-PAGE, and immunoblot analysis was carried out.

**In Vitro Pulldown Assay**—WT NEIL1 or its truncation mutants (20 pmol) were incubated with His-tagged PCNA (10 pmol) in 0.5 ml of Tris-buffered saline for 1 h at 4 °C and then mixed with His-Select HC nickel beads (Sigma) with constant rotation for 1 h at 4 °C. The beads were subsequently washed extensively with 50 mM Tris-HCl (pH 7.5) and 500 mM NaCl. After elution of the proteins with SDS sample buffer and SDS-PAGE, the presence of NEIL1 was tested by immunoblotting.

GST pulldown assays were performed as described previously (33). Briefly, proteins were mixed with glutathione–Sepharose beads (20 μl) alone or bound to GST-tagged truncated NEIL1 domains (positions 312–349 and 312–389; 10 pmol) and incubated with PCNA (2.5 pmol) in 0.5 ml. After washing, the bound proteins were separated by SDS-PAGE, and the presence of PCNA was tested by immunoblotting.

**Far Western Analysis**—The interacting proteins (40 pmol) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. All subsequent steps were performed as described previously (29) with only slight modifications. The membrane was probed with PCNA (10 pmol/ml) in phosphate-buffered saline supplemented with 0.5% nonfat dried milk, 0.05% Tween 20, 10 mM trimethylamine N-oxide, and 1 mM dithiothreitol for 4 h, followed by washing. Immunoblot analysis was then performed to detect the presence of PCNA.

**Mammalian Two-hybrid Analysis**—WT NEIL1 was cloned into the pCMV-BD vector containing five tandem repeats of the Gal4-binding sites (pFR-Luc; Stratagene) to yield pCMV-NEIL1BD, which encodes a Gal4-NEIL1 fusion protein. WT PCNA was cloned into the pCMV-AD vector to yield pCMV-PCNAAD, which contains the transcriptional activation domain of mouse NF-κB (Stratagene) and encodes a PCNA fusion protein. HCT116 cells were cotransfected with pCMV-NEIL1BD and pCMV-PCNAAD or an equivalent amount of the empty vector; pCMV-β-gal was used as an internal standard. Luciferase activity was measured in a luminometer using a luciferase assay kit (Promega) at 48 h after transfection and normalized with coexpressed β-galactosidase activity.

**Electrophoretic Mobility Shift Assay (EMSA)**—The 5'-32P-labeled 5-OHU-containing 51-mer oligonucleotide and a control oligonucleotide of identical sequence except for substitution of 5-OHU with Cyt at position 26 (see Table 1) were used. The DNA (15 fmol) was then incubated with NEIL1 (5 nM) and various amounts of PCNA for 10 min at 22 °C in buffer containing 40 mM HEPES (pH 7.3), 50 mM KCl, 12% glycerol, and an appropriate amount of BSA to maintain an equal amount of total protein in each reaction. After electrophoresis on non-denaturing 10% polyacrylamide gels in Tris/glycine buffer (pH 8.4), the protein-DNA complex was quantitated in a PhosphorImager using ImageQuant software.
Surface Plasmon Resonance (SPR) Analysis of NEIL1-DNA Binding—The effect of PCNA on the interaction between NEIL1 and bubble DNA was analyzed by SPR using Biacore X (GE Healthcare). 5′-Biotinylated bubble oligonucleotide (see Table 1) was bound to Sensor Chip SA according to the manufacturer’s instructions. Interaction analysis was carried out using HEPES-buffered saline containing 50 mM EDTA and 0.005% Tween 20 with NEIL1 alone or together with PCNA. The analyte solution was passed over the sensor chip at 20 μl/min, and the response units were corrected for the blank reading. Regeneration buffer (HEPES-buffered saline with 350 mM EDTA and 0.005% Tween 20) was injected at 20 μl/min to regenerate the surface in between analysis cycles. This procedure did not reduce binding of the sensor chip surface.

Kinetic constants were calculated from the sensorgrams using the BIAevaluation software (Version 3.1, Biacore) and a global fitting model. Response curves were prepared for fitting by subtraction of the signal generated simultaneously on the BSA control flow cell and then globally fitted to a bivalent analyte model (first step, A + B ↔ AB, and second step, AB + B ↔ AB₂, where A = ligand and B = analyte).

RESULTS

NEIL1 Association with PCNA in Vivo—Our previous observation of the unique activity of NEIL1 on single-stranded and bubble DNA substrates and of its S phase-specific activation suggested linkage of the in vivo function of NEIL1 to DNA replication (31). Because of the established role of PCNA as a scaffold and recruitment site during DNA replication and repair, we tested for in vivo association between NEIL1 and PCNA by performing co-immunoprecipitation of extracts from HEK293 cells expressing either the NEIL1-FLAG or NEIL2-FLAG polypeptide. We tested NEIL2 as well because it shares with NEIL1 a preference for single-stranded region-containing substrates, but, unlike NEIL1, is not cell cycle-regulated. PCNA was found to be present in both NEIL1 and NEIL2 immunoprecipitates but to a greater extent in the NEIL1 immunoprecipitate (Fig. 1A, lanes 2 and 4). PCNA was not found in the empty FLAG vector control (Fig. 1A, lane 1).

Furthermore, to address the possibility that the presence of contaminating DNA contributed to PCNA being detected in the NEIL1 immunoprecipitates, we treated the cell lysate with DNase I. DNase treatment removed most contaminating genomic DNA (supplemental Fig. 1). The removal of DNA did not diminish the amount of PCNA present in either the NEIL1 or NEIL2 immunocomplex (Fig. 1A, lanes 3 and 5), suggesting that DNA is not required for the stable association of NEIL proteins with PCNA. Immunoblot analysis using an anti-FLAG antibody demonstrated that equal amounts of NEIL1-FLAG or NEIL2-FLAG were present in both untreated and DNase-treated samples. In a similar experiment, we were also able to detect the 140-kDa subunit of replication factor C (RFC) in the NEIL1-FLAG complex (supplemental Fig. 2, lane 1). This subunit is unique to the RFC complex that serves as the clamp loader for loading the PCNA clamp onto primed templates. RFC could not be detected when the cells were transfected with the empty FLAG vector (supplemental Fig. 2, lane 2). Although these results do not establish binary interaction between PCNA and NEIL1, they strongly suggest that endogenous NEIL1 and PCNA are present in the same in vivo complex even in the absence of DNA. This prompted us to test for direct interaction of NEIL1 with PCNA.

Mapping the Interaction Domain of NEIL1—We showed previously that NEIL1 interacts with several proteins involved in SN-BER, including DNA ligase IIIα, pol β, and XRCC1 (29).
TABLE 1
Sequences of 5-OHU-containing oligodeoxynucleotides

| SS DNA Oligo | 5'-GCT TAG CTT GGA ATG GTA TCA TGA TTA GAA CTC GTG TGC GCT GTA GAC GAC GTC GGC -3' |
|--------------|------------------------------------------------------------------------------------|
| Duplex Oligo | 5'-GCT TAG CTT GGA ATG GTA TCA TGA TTA GAA CTC GTG TGC GCT GTA GAC GAC GTC GGC -3' |
| Bubble (11-nt) Oligo | 5'-GCT TAG CTT GGA ATG GTA TCA TGA TTA GAA CTC GTG TGC GCT GTA GAC GAC GTC GGC -3' |
| 5' Fork Oligo | 5'-GCT TAG CTT GGA ATG GTA TCA TGA TTA GAA CTC GTG TGC GCT GTA GAC GAC GTC GGC -3' |
| 3' Fork Oligo | 5'-GCT TAG CTT GGA ATG GTA TCA TGA TTA GAA CTC GTG TGC GCT GTA GAC GAC GTC GGC -3' |

The interaction domain of NEIL1 for all of these proteins is localized within residues 288–349 in the C terminus (29). To test the possibility that the same region also includes the binding site for PCNA, we used His tag (Fig. 1B) and GST tag (Fig. 1D) pulldown assays and Far Western analysis (Fig. 1C).

We confirmed that NEIL1 and PCNA directly interact, and that the interaction domain of NEIL1 is localized within the C-terminal domain. Fig. 1B shows that His-tagged PCNA interacted with full-length and CA40 NEIL1, but not with the CA101 truncation mutant (upper panel, lanes 3–5). We also fused NEIL1 C-terminal regions to GST and showed that PCNA interacted with this fusion protein (Fig. 1B, lanes 6 and 7), demonstrating that the NEIL1 interaction domain alone is sufficient for stable binding to PCNA. Used as a control, GST alone did not interact with PCNA (Fig. 1B, lane 8). In a reciprocal pulldown assay, we used the GST-tagged NEIL1 domains to confirm that the C-terminal interaction domain of NEIL1 alone was sufficient for interaction. Indeed, C-terminal fragments of NEIL1 in the absence of the rest of the protein were sufficient for stable interaction with PCNA (Fig. 1D, lanes 3 and 4). Again, GST itself was unable to interact with PCNA (Fig. 1D, lane 2).

These results were confirmed by Far Western analysis in which PCNA in solution was incubated with various membrane-immobilized proteins (Fig. 1C, right panel). Once again, deletion of 101 C-terminal residues of NEIL1 abolished interaction, and the C-terminal domain fused to GST was sufficient for interaction. We immobilized GST and BSA as controls (Fig. 1C, lanes 7 and 8), and they did not show interaction. FEN1, whose interaction with PCNA is well established, was used as a positive control (Fig. 1C, lane 9). In a reciprocal Far Western analysis, we could not detect interaction when PCNA was present on the membrane after SDS-PAGE (data not shown). This was perhaps expected because denatured PCNA on the gel was unlikely to refold to the native trimeric structure after the renaturation procedure needed for binding to NEIL1.

Table 1: Sequences of 5-OHU-containing oligodeoxynucleotides

The table lists various oligodeoxynucleotides containing 5-OHU, along with their corresponding sequences.

PCNA Stimulates NEIL1 in a DNA Structure-specific Manner—We examined the activities of NEIL1 and NEIL2 in the presence of PCNA using oligonucleotide substrates of several structures containing duplex and single-stranded regions, specifically fork structures with the lesion placed 3’ or 5’ of the branch point (Table 1). We compared the effect of PCNA on the activities of NEIL1 and NEIL2, both of which are able to excise the same base lesion (5-OHU) from similar substrate structures (31), and PCNA was present in the immunocomplexes of both enzymes (Fig. 1A). Only slight stimulation of NEIL1 activity was observed on duplex DNA in the presence of PCNA, but significant enhancement of NEIL1 activity was observed when the lesion was located in a bubble or single-stranded oligonucleotide or in the single-stranded region near the junction of a fork oligonucleotide (Fig. 2). PCNA enhancement of NEIL1 activity was comparable for oligonucleotide substrates with a 3’-versus 5’-fork (Fig. 2). A similar increase in activity was also observed with fork substrates when the lesion in the single-stranded region was placed as far away as 10 nucleotides away from the fork junction (data not shown). In contrast to the results with NEIL1, NEIL2 activity with all substrates was barely affected by the presence of PCNA (Fig. 2). An earlier report described a similar finding with NTH1, which also excises 5-OHU, but only from duplex DNA; NTH1 was shown to bind to PCNA, but was not stimulated as a result of such binding (34). We also showed a concentration dependence of NEIL1 stimulation by PCNA for all substrates. The extent of stimulation was highest with the 3’-fork substrate, intermediate for single-stranded or bubble substrate, and negligible for the duplex DNA (Fig. 3A). PCNA stimulation of NEIL1 activity could be due to its ability to load NEIL1 on the lesion site when PCNA is concentrated at the fork junction of a partially duplex DNA (e.g., a primed template). PCNA could also alter NEIL1 conformation, thus facilitating its substrate binding on single-stranded lesions. Again, no significant stimulation of NEIL2 activity was observed in the presence of increasing concentrations of PCNA, which confirmed the results in Fig. 2.

We then determined the kinetic parameters of NEIL1 activity on a bubble DNA substrate in the presence or absence of PCNA using conditions for single turnover in which the enzyme is present in excess, and product formation follows first-order kinetics (35). The data were analyzed using the first-order rate equation [P] = A0(1 − exp(−kobs*t)), where A0 represents the amplitude of the exponential phase and kobs is the rate constant correlated with the reaction (Fig. 3B). For NEIL1 alone, the kobs was 0.012 ± 0.002 s−1, and with PCNA, it was 0.033 ± 0.002 s−1. Because kobs = kobs[S0], it is evident that PCNA stimulates the initial rate of damaged base removal by NEIL1.

Effect of PCNA on the Affinity of NEIL1 for Bubble DNA—Enhancement of NEIL1 activity by PCNA with the bubble substrate suggested that NEIL1 has intrinsic affinity for the single-stranded DNA. We tested this using EMSA and surface plasmon resonance. We used a control oligonucleotide whose sequence was identical to that of the 5-OHU-containing oligonucleotide except for the substitution of 5-OHU with Cyt. We could not carry out the binding studies with the lesion-contain-
ing DNA because the activity of WT NEIL1 could not be inhibited during execution of the experiment. Fig. 4A shows a representative analysis of the WT NEIL1-DNA complex in the presence or absence of PCNA. PCNA showed no direct binding to the DNA oligonucleotide. We observed a second slower migrating complex with bubble DNA that appeared to be due to the binding of two NEIL1 molecules to a single oligonucleotide, which became more pronounced with increasing protein concentration. NEIL1 had no excision activity on these oligonucleotides lacking an oxidatively damaged base, as expected (data not shown).

We observed an increase in NEIL1 affinity for DNA in the first step binding, and a 2.7-fold increase in $K_{d}$ was shown on the bubble substrate in the presence of PCNA (Fig. 4B and Table 2). A similar trend was also observed when we used the catalytically inactive NEIL1(K53L) mutant with the 5-OHU-containing substrate (data not shown).

We utilized SPR to examine the real-time kinetics of NEIL1 binding to bubble DNA as described above (Fig. 5). Table 2 shows that the NEIL1-PCNA complex had ~2-fold higher affinity than NEIL1 alone for the bubble oligonucleotide. The binding constants calculated from EMSA and SPR studies are not identical, presumably because of the different ionic strengths of the reaction mixtures used in these experiments. The low salt concentration used in EMSA could not be used in the SPR studies because of nonspecific binding to the sensor chip. Taken together, these data show that PCNA increases the affinity of NEIL1 for the substrate and thus its enzymatic activity and that NEIL1 has intrinsic affinity for single-stranded regions in bubble and fork DNA structures.

**Mammalian Two-hybrid Analysis for in Vivo NEIL1-PCNA Interaction**—Using a mammalian two-hybrid assay system, we analyzed the in vivo interaction between NEIL1 and PCNA (Fig. 6). A major advantage of this system is that, unlike with *E. coli* or yeast two-hybrid systems, interaction of proteins occurs in the native environment where these interacting partners could be covalently modified. In this system, the Gal4 DNA-binding domain fused to NEIL1 (pCMV-NEIL1BD) binds to its cognate site in the Gal4-containing promoter, thus directly recruiting NEIL1 to the promoter. A functional transcriptional activator is created by bringing the NF-κB activation domain that is fused to PCNA (pCMV-PCNAAD) in close proximity to the Gal4-binding domain, which is accomplished when NEIL1 interacts with PCNA. HCT116 cells were cotransfected with these plasmids. Simultaneous expression of pCMV-NEIL1BD and pCMV-PCNAAD enhanced luciferase reporter activity by >7-fold compared with expression of Gal4 (pCMV-Gal4) alone (Fig. 6, bar 1 versus bar 4). Although the stimulation was modest, this could be explained by the competition of ectopic PCNA with the endogenous protein. No increase in luciferase activity was observed when either pCMV-NEIL1BD or pCMV-PCNAAD was transfected alone (Fig. 4, bars 2 and 3). These results further confirm that NEIL1 and PCNA interact in vivo.

**NEIL1 Interacts with the Interdomain Connector Loop of Human PCNA**—Because of our characterization that the PCNA-interacting sequence is located in the unconserved C-terminal region of NEIL1, we looked for the presence of the canonical PCNA-interacting sequence (PIP box) common in many PCNA partners. Such a motif is absent in NEIL1; however, we identified a sequence that is nearly identical to the N4 region present in the 125-kDa subunit of mammalian pol δ (36). The alignment of NEIL1 residues 296–311 with the N4 region present in the 125-kDa subunit of mammalian pol δ is shown in Table 3, demonstrating sequence conservation between the two, specifically the KATQ sequence.

![FIGURE 2. Effect of PCNA on NEIL1 and NEIL2 for 5-OHU excision from various DNA structures.](image_url)
domain, connecting the two domains in PCNA monomers. This sequence (called the interdomain connector loop) is involved in the direct interaction of PCNA with many proteins, including human pol H9254 (37). Site-directed mutagenesis of a single amino acid residue (Gly127) to Ala in this loop of PCNA significantly reduced this interaction (37). We tested whether the G127A mutation also affects NEIL1 interaction with PCNA using the mammalian two-hybrid system. The G127A mutant significantly reduced the affinity of NEIL1 for PCNA (Fig. 6, bar 5). This suggests that the interdomain connector loop of PCNA is important for interaction with the C terminus of NEIL1.

**DISCUSSION**

Our observation that, unlike the other three mammalian DNA glycosylases (OGG1, NTH1, and NEIL2) specific for repair of oxidized bases, NEIL1 shows strong S phase-specific activation provided the rationale for investigating the interaction of NEIL1 with PCNA. We (31) and others (15, 16, 18) have postulated preferential repair of oxidized bases in replicating cell genomes based on the reasoning that bulky base adducts induce persistent strand breaks by blocking chain elongation, which may activate signals for repair. In contrast, most oxidized base adducts allow replication (and transcription) to proceed past the damaged site and could thus induce mutations. Therefore, although the signaling for repair of such damage could be subtle, the repair should still be extremely urgent.

PCNA is a homotrimeric ring with 6-fold symmetry because of the presence of two equivalent domains in each subunit (38). The central role of PCNA in DNA transactions is to act as a platform for recruiting the proteins involved in DNA metabolism. During replication, it is loaded by RFC at the primer-template junction of the growing chain, increasing the processivity of pol H9254 while activating FEN1 and DNA ligase I via binary interactions (39). In view of the trimeric nature of PCNA, it was suggested that each subunit may form a complex with distinct replication or repair proteins. PCNA also activates translesion synthesis DNA poly-

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**FIGURE 3.** PCNA dose-dependent stimulation of NEIL1 activity and single turnover kinetics. A, the fold increase in activity (activity<sub>NEIL-PCNA</sub> − activity<sub>NEIL</sub>/activity<sub>NEIL</sub>) is plotted for NEIL1 (3 nM) and NEIL2 (6 nM) with duplex (upper left), bubble (upper right), single-stranded (lower left), and 3'-fork (lower right) DNA structures (all at 25 nM) as a function of PCNA concentration. B, shown is the effect of PCNA (0.5 μM) on single turnover kinetics of NEIL1 (20 nM) using 5-OHU-containing bubble oligonucleotide (2 nM) substrate.
neocomplex contains PCNA, but is not stimulated by PCNA. NTH1, another oxidized base-specific DNA glycosylase, was also shown to interact with PCNA but without activation (34). Two other DNA glycosylases, viz. uracil-DNA glycosylase (UNG2) and 8-oxo-Gua-Ade-specific adenine-DNA glycosylase (MYH), were also shown to be stimulated by PCNA (41). UNG2 was suggested to have a preferential activity on repairing misincorporated Ura in nascent DNA (42). A recent study documented the presence of many replicative proteins in the UNG2 immunocomplex (43). MYH is similarly responsible for removing misincorporated Ade opposite 8-oxo-Gua in the template strand (44). This provides an opportunity to prevent mutation due to unrepaired 8-oxo-Gua in the template DNA. Thus, repair by UNG2 and MYH should be nascent strand-specific and further supports replication-associated BER (45).

Many PCNA partners, including MYH and UNG2, interact via the consensus PIP motif (46, 47). In the case of the NEIL1 polypeptide lacking this motif, we have identified the interaction domain to be in the C terminus, which contains a sequence not entirely conserved in mammalian NEIL1 but conserved in mammalian pol δ (36). The interaction involving this region appears to be weaker than that for the PIP motif. Although the PIP motif was the first to be discovered for the PCNA-binding interface, it now appears, consistent with the plethora of PCNA-interacting partners, that other peptide sequences may also be involved in binding to this sliding clamp (39). For example, the human translesion synthesis pol λ lacks the PIP motif, but binds to PCNA (48). On the other hand, a sequence in the interdomain linker region of PCNA appears to be the common interacting interface for its partners (39). We confirmed that this region containing Gly127 is also involved in interaction with NEIL1 because the G127A mutant showed reduction in binding to and activating NEIL1 with a fork substrate.

The PCNA sliding clamp lacking affinity for DNA is loaded in topologically constrained duplex DNA by the clamp loader RFC. In studies using linear duplex DNA with unblocked termini, RFC was not shown to be required for loading PCNA, which could presumably slide on and off DNA using the ends. PCNA could thus maintain a steady-state equilibrium between the free and sliding fractions when present in excess. However, it is interesting that we observed the presence of RFC along with PCNA in the NEIL1 immunocomplex, suggesting that NEIL1 may also have direct interaction with the clamp loader as well.

Our observation of the NEIL1-PCNA complex raises the issue of stoichiometry of the PCNA complex formed in vivo. Although we will attempt to address this difficult question in the future, we propose a simple-minded scenario of the role of NEIL1 in repairing oxidized bases during DNA replication. In this model, the replication complex contains NEIL1, which either could be recruited upon encountering an oxidized base lesion or could be an intrinsic component of the complex. After unwinding of the template ahead of the growing chain, the single-stranded template is complexed with replication protein A, the single-stranded DNA-binding protein, which protects the DNA from degradation. We propose that PCNA-bound NEIL1 initiates repair of the oxidized base by recognizing the oxidative lesion site in either the leading or lagging strand template ahead.

**TABLE 2**

Affinity constants of the NEIL1-PCNA complex on bubble DNA in EMSA (A) and kinetic parameters and affinity constants of the NEIL1-PCNA complex on bubble DNA in SPR analysis (B)

|         | $K_{a1}$  | $K_{a2}$  |
|---------|-----------|-----------|
| NEIL1   | 32.2 ± 2.7| 2.6 ± 0.3 |
| NEIL1 + PCNA | 20.0 ± 1.2| 7.1 ± 0.1 |

|         | $K_{off1}$ | $K_{off2}$ | $K_{on1}$ | $K_{on2}$ | $K_{off}$ | $K_{on}$ |
|---------|------------|------------|-----------|-----------|-----------|---------|
| NEIL1   | 2.7 × 10^5 | 3.1 × 10^5 | 1.5 × 10^6| 1.0 × 10^6| 8.2 × 10^4| 9.2 × 10^4|
| NEIL1 + PCNA | 6.0 × 10^4 | 7.0 × 10^4 | 1.0 × 10^5| 1.0 × 10^5| 1.0 × 10^5| 1.0 × 10^5|

*RU, response units.*

merases, including pol η, when the replication complex stops at a noninstructional bulky adduct in the template (40). The polymerase switching allows the translesion synthesis polymerase to insert a base opposite the noninstructional adduct to get past the block, followed by the second switching to allow the replication complex to resume copying of the undamaged template.

Our previous observation that NEIL1 is cell cycle-regulated strongly suggested that NEIL1 has a unique role in repair of replicating DNA and that it may have functional interactions with proteins of the replisome. This was further supported by our following results: 1) PCNA preferentially stimulates NEIL1 with single-stranded or fork DNA substrates; and 2) NEIL2 is also active on single-stranded DNA substrates, and its immu-
of the polymerase. The strand interruption prevents further movement of the fork, which then collapses to form a “chicken foot” structure (49). Repair of the damage occurs in the reannealed duplex DNA, and replication resumes after a helicase, e.g. Werner or Bloom protein, resolves the collapsed fork (50). In support of this scenario, we have recently shown that NEIL1 stably interacts with and is activated by the Werner protein (33). How the steps are coordinated in this process is obscure. Nevertheless, it appears that the repair-replication complex is dynamic with the likelihood of coordinated handover among the interacting partners (39). It is tempting to speculate that the weak interaction between PCNA and NEIL1 is needed for the efficient release of NEIL1 after completion of repair.
Most studies of base damages imply that their repair occurs in nonreplicating genomes, which contain only duplex structure. Such repair involves either single nucleotide incorporation in the SN-BER pathway or long patch base excision repair (LP-BER) synthesis by using PCNA and FEN1 together with pol\(\beta\) and DNA ligase I. Earlier studies confirmed the possibility of LP-BER in vitro for repair of synthetic or reduced AP sites that could not be repaired via pol\(\beta\)-dependent SN-BER (52, 53). APE1 cleaves the AP site as well as its reduced form or tetrahydropyrano, which mimics the AP site (54). Interaction of APE1 with PCNA is consistent with the involvement of PCNA in LP-BER (55). More recent studies showed that LP-BER could also be mediated by pol\(\beta\), where FEN1 could remove the 5'-terminal region instead of the lyase activity of pol\(\beta\) (56). Stable interaction of pol\(\beta\) with FEN1 and PCNA strongly suggests that LP-BER could be mediated by both pol\(\beta\) and pol\(\delta\), whereas SN-BER uniquely requires pol\(\beta\). We have shown previously that NEIL1 stably interacts with pol\(\beta\) as well as XRCC1 and DNA ligase III\(\alpha\) to carry out SN-BER (29). The interaction of NEIL1 with PCNA documented in this study suggests that NEIL1 may also be involved in APE1-independent LP-BER.

PCNA has recently been shown to be monoubiquitylated in response to UV light, which enhances its interaction with and activation of pol\(\eta\) (57). PCNA was previously shown to interact with p300/CBP (cAMP-responsive element-binding protein–binding protein) histone acetyltransferase and may be acetylated (51, 58). Whether such covalent modifications of PCNA and NEIL1 modulate their interaction remains to be established.

Finally, 9-1-1, a heterotrimeric complex of Rad9, Rad1, and Hus1, is an alternative sliding clamp in mammalian cells that is...
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distinct from PCNA and activated by DNA damage and cell cycle checkpoints. Guan et al. have recently shown a stable interaction of NEIL1 with 9-1-1 and its stimulation as a result of such interaction (32). The presence of multiple complexes of NEIL1 underscores the need for developing a comprehensive picture of the involvement of NEIL1 (and possibly other DNA glycosylases) in various subpathways of BER in response to both endogenous and induced oxidative damage in the genome and of the linkage between BER, damage signaling, and DNA metabolic pathways.

Acknowledgments—We thank Thomas Wood (Director of the Molecular Genetics Core) and Alex Kurosky and Steven Smith (Protein Chemistry Core Laboratories, University of Texas Medical Branch) for various services and analysis, David Konkel for critical editing, and Wanda Smith for expert secretarial assistance.

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