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NEIL1 excises 3′ end proximal oxidative DNA lesions resistant to cleavage by NTH1 and OGG1

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ABSTRACT

Base excision repair is the major pathway for the repair of oxidative DNA damage in human cells that is initiated by a damage-specific DNA glycosylase. In human cells, the major DNA glycosylases for the excision of oxidative base damage are OGG1 and NTH1 that excise 8-oxoguanine and oxidative pyrimidines, respectively. We find that both enzymes have limited activity on DNA lesions located in the vicinity of the 3′ end of a DNA single-strand break, suggesting that other enzymes are involved in the processing of such lesions. In this study, we identify and characterize NEIL1 as a major DNA glycosylase that excises oxidative base damage located in close proximity to the 3′ end of a DNA single-strand break.

INTRODUCTION

The generation of reactive oxygen species (ROS) through oxidative metabolism and ionizing radiation produces a variety of lesions in DNA, such as oxidized bases and strand breaks. When found isolated, the majority of base lesions in DNA are repaired by the base excision repair (BER) pathway that is initiated by the excision of the damaged base by a DNA glycosylase (1). However, oxidative lesions, including oxidized bases and DNA single-strand breaks (SSBs), may arise in close proximity to each other as ROS can be produced at high local concentrations through the deposition of ionizing radiation energy in small volumes of nanometre dimensions (2). Although still performed by BER, the repair of these so-called 'clustered lesions' is a more complex process since the neighbouring lesions affect each other during repair (3–5).

Two major DNA glycosylases are involved in recognition and excision of oxidative base lesions in human cells. 8-oxoguanine-DNA glycosylase (OGG1) protein is the major DNA glycosylase involved in the excision of 8-oxoguanine (8-oxoG) (6,7), and endonuclease III (NTH1) protein is the major DNA glycosylase involved in the excision of oxidized pyrimidines, such as 5-hydroxyuracil (5-OHU), thymine glycol and 5,6-dihydrouracil (8,9). Recently, the *Ogg1* and *Nth1* gene knockout revealed a back-up repair activity for oxidative DNA lesions (10,11) later attributed to the endonuclease VIII (Nei)-like proteins (NEIL) that possess a broad substrate specificity (12,13). The preferred substrates for excision by human NEIL1 include 5-OHU, 5-hydroxycytosine, formamidopyrimidine derivatives of A and G (FapyA and FapyG) and thymine glycol. However, the activity of NEIL1 against 8-oxoG was found to be very weak in comparison to other substrates and more specific for an 8-oxoG:G mispair (12–14).

Several reports documented limited activity of the major DNA glycosylases (NTH1 and OGG1) on oxidative base lesions located at the 3′-termini of SSB (15,16), however the mechanism involved in repair of oxidative DNA lesions located in close proximity to the 3′ end have not been identified. In the present work, we have used purified DNA glycosylases and oligonucleotide duplexes containing 8-oxoG and 5-OHU at different positions in the vicinity of a SSB and identify NEIL1 as a major enzyme involved in excision of the 3′ end proximal lesions.

MATERIALS AND METHODS

Materials

Synthetic oligodeoxyribonucleotides were purchased from Eurogentec and purified by electrophoresis on a 20% polyacrylamide gel. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from PerkinElmer Life Sciences. His-tagged OGG1 and NTH1 were purified on a nickel chelating resin (Novagen) as recommended by the manufacturer. Full-length native NEIL1 was purified as described (17).

Substrate labelling

Oligonucleotides containing 8-oxoG or 5-OHU were 5′ end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase and unincorporated label was removed on a Sephadex G-25 spin column. To prepare the substrates, the labelled 8-oxoG or

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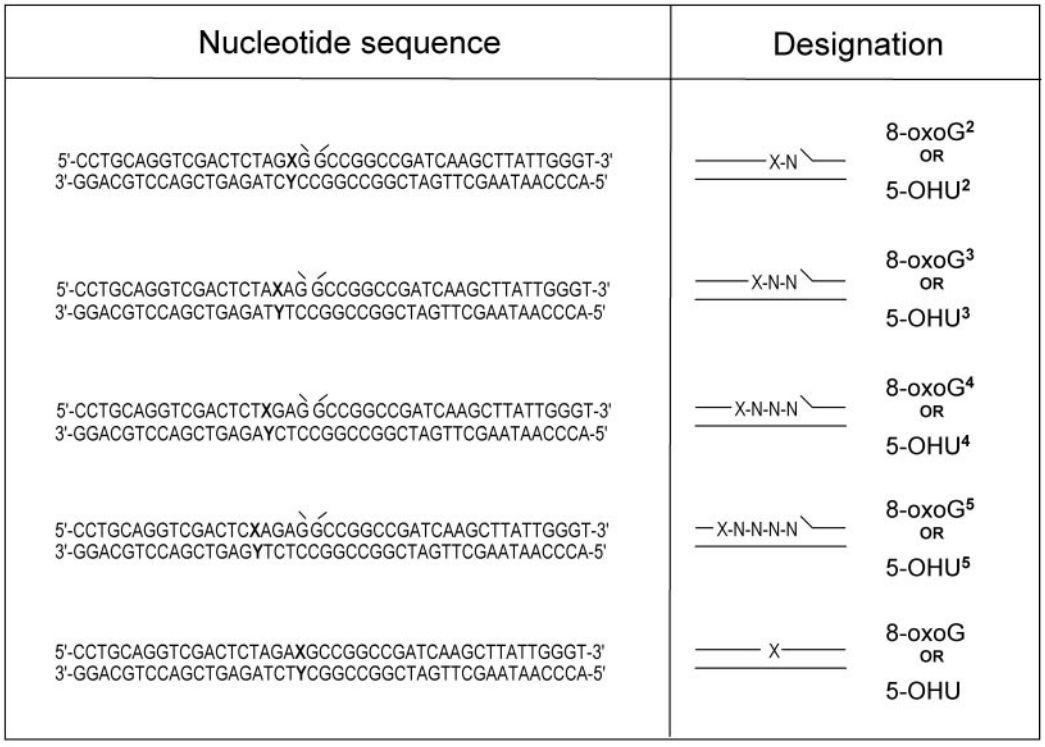

Figure 1. Structures of oligonucleotides used. Oligonucleotides (20mer) containing 8-oxoG or 5-OHU (designated X) were 5′ end-labelled and a 24mer adjacent oligonucleotide added and annealed to the corresponding complementary strand (with base Y corresponding to cytosine and guanine opposite to 8-oxoG and 5-OHU, respectively) to generate substrates containing 8-oxoG or 5-OHU located 1–4 nt apart from a DNA single-strand break. Substrates containing 5′ end-labelled isolated 8-oxoG or 5-OHU were also used as a control.

5-OHU containing oligonucleotides were annealed to the relevant oligonucleotides shown in Figure 1 at 90°C for 3–5 min followed by slow cooling to room temperature.

Excision assays

Assays contained 300 fmol oligonucleotide per reaction in 10 µl of reaction buffer containing 50 mM HEPES–KOH (pH 7.8), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 1.5 mM DTT, 8.5% glycerol and 100 µg/ml BSA, and the indicated amount of OGG1, NTH1 or NEIL1. All reactions were incubated for 20 min at 37°C, and where stated, mixed with an equal volume of 10% piperidine, the samples heated to 95°C for 15 min to cleave the abasic sites produced during the enzymatic reaction and dried. Formamide loading dye (95% formamide, 0.02% xylene cyanole, 0.02% bromophenol blue) was added, the samples were heated to 95°C for 5 min and the products separated by 20% denaturing polyacrylamide gel electrophoresis in 1× TBE. The gels were subsequently exposed to a storage phosphor screen at 4°C prior to analysis by phosphorimaging. To calculate enzyme kinetic parameters, the proteins were incubated with varying amounts of substrate (30–600 fmol) for 10 min at 37°C, the gels were quantified using Quantity One software and Lineweaver–Burke plots produced.

Electrophoretic mobility shift assay (EMSA)

Assays contained 300 fmol oligonucleotide and the indicated amount of either OGG1 or NEIL1 per reaction in 20 µl of buffer containing 20 mM Tris–HCl (pH 7.5), 100 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 20 µg/ml BSA, 7.5% glycerol and 0.1% Triton X-100 for 15 min on ice. An aliquot of 5 µl of loading dye (30% glycerol, 0.25% xylene cyanol, 0.25% bromophenol blue) was added and the samples were analysed on a 12% non-denaturing polyacrylamide gel at 4°C in 0.5× TBE. The gels were dried and exposed to storage phosphor screens prior to analysis by phosphorimaging.

All experiments were done in triplicate and representative gels are shown.

RESULTS

Excision of 5-hydroxyuracil located near a DNA single-strand break by NTH1 and NEIL1

In mammalian cells, both NTH1 and NEIL1 DNA glycosylases are involved in the excision of oxidized pyrimidines (8,9,12,13). To address the role of these enzymes in the repair of oxidative DNA lesions located near DNA SSB, oligonucleotide substrates containing 5-OHU in different positions proximal to a SSB were generated (Figure 1). The 5-OHU lesion containing strand was 5′ end-labelled with ^{32}P to monitor the excision of the modified base. It has been recently demonstrated that 5,6-dihydrouracil on the 3′-termini of a DNA SSB, which is resistant to excision by NTH1, is excised by human AP endonuclease (APE1) (15). In agreement with these data, we found that 5-OHU located at the 3′-termini is also resistant to excision by NTH protein, but can be excised by APE1 (data not shown). We further demonstrate that although both NTH1 and NEIL1 can excise isolated 5-OHU

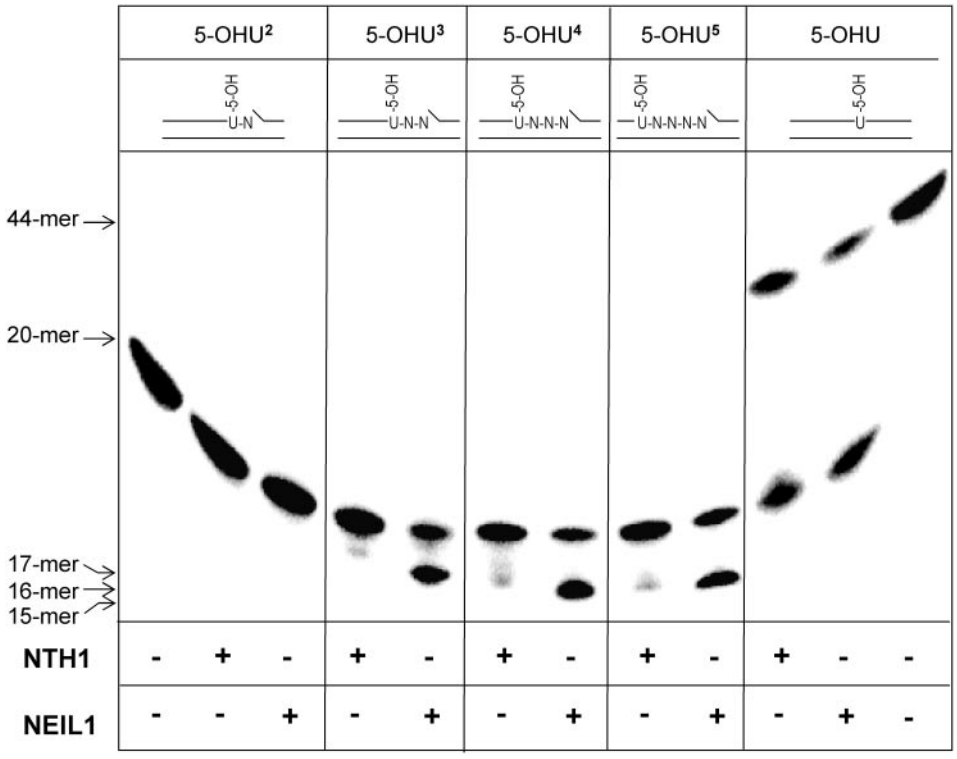

Figure 2. Comparison of the excision of 5-OHU located near a DNA single-strand break by NTH1 and NEIL1. Oligonucleotide substrates (0.3 pmol), shown at the top of each panel, were incubated with either NTH1 (0.6 pmol) or NEIL1 (0.5 pmol) for 20 min at 37°C and formamide loading dye added. An aliquot was analysed by 20% denaturing PAGE and phosphorimaging. The first and last lane contains a size marker for untreated oligonucleotide substrate.

(Figure 2; far right panel), neither NEIL1 nor NTH1 are able to efficiently excise 5-OHU located as a second nucleotide 5′-upstream to the SSB (5-OHU^2, Figure 2). Interestingly, if the 5-OHU lesion is moved further 5′-upstream to the SSB, NTH1 has minimal excision activity on the lesion, however NEIL1 retains its activity on all three of the substrates tested and is comparable to the activity observed on DNA containing isolated 5-OHU. No piperidine cleavage of AP sites was performed prior to electrophoresis, as both NEIL1 and NTH1 have robust β-lyase activity. However, even with piperidine treatment no excision of 5-OHU located in close proximity to a SSB was observed after NTH1 treatment (data not shown) indicating that the DNA glycosylase activity and not the β-lyase activity of NTH1 is inhibited by the presence of the SSB.

Excision of 8-oxoguanine located near a DNA single-strand break by OGG1 and NEIL1

In mammalian cells, excision of isolated 8-oxoG in the context of an 8-oxoG:C pair is mainly accomplished by OGG1 (6,7). It was suggested that NEIL1 may also be involved in repair of 8-oxoG, although the activity of NEIL1 on this lesion is relatively weak (12,13). To address the role of these enzymes in the repair of 8-oxoG located near a DNA SSB, oligonucleotide substrates containing 8-oxoG in different positions proximal to a SSB were generated (Figure 1). We have previously demonstrated that both NEIL1 and OGG1 were unable to excise 8-oxoG that is located at the very 3′-terminus of a SSB and that it is removed by the phosphodiesterase activity of APE1 (16). In this study, we found that neither NEIL1 nor OGG1 are able to efficiently excise 8-oxoG located as a second nucleotide 5′-upstream to the strand break (8-oxoG^2, Figure 3) and this is similar to the lack of activity observed with NEIL1 and NTH1 on 5-OHU in the same position. However, if the 8-oxoG lesion is moved one nucleotide further 5′-upstream (8-oxoG^3), then surprisingly NEIL1 is able to efficiently remove the lesion, but OGG1 activity on this substrate is limited. Furthermore, if the 8-oxoG lesion is moved one more nucleotide further upstream (8-oxoG^4), then OGG1 is able to efficiently remove the lesion, although NEIL1 also has activity against this substrate and thus may serve as a back-up repair system. If the 8-oxoG lesion is placed as the fifth nucleotide from the strand break (8-oxoG^5), NEIL1 becomes less efficient at the removal of the lesion, while OGG1 retains full activity.

Unlike 5-OHU, the activity of NEIL1 on 8-oxoG^3 and 8-oxoG^4 was dramatically increased in comparison with the weak activity observed on isolated 8-oxoG within DNA [(12,13) and Figure 6]. Subsequently, we decided to calculate kinetic parameters of OGG1 and NEIL1 for these substrates. Comparing the kinetic data of OGG1 for the excision of 8-oxoG^3 and 8-oxoG^4, it is apparent that OGG1 has a similar K_m for both substrates but a 10-fold lower catalytic turnover (k_{cat}) for 8-oxoG^3 (Table 1). Our kinetic parameters for an isolated 8-oxoG:C substrate are similar to previously published data for excision of 8-oxoG within DNA [(18), Table 1]. In comparison, NEIL1 has an ~7-fold lower K_m than OGG1 for excision of 8-oxoG^3, although the turnover of these enzymes are very similar. The K_m and k_{cat} values of NEIL1 for excision of 8-oxoG^3 and 8-oxoG^4 are also similar,

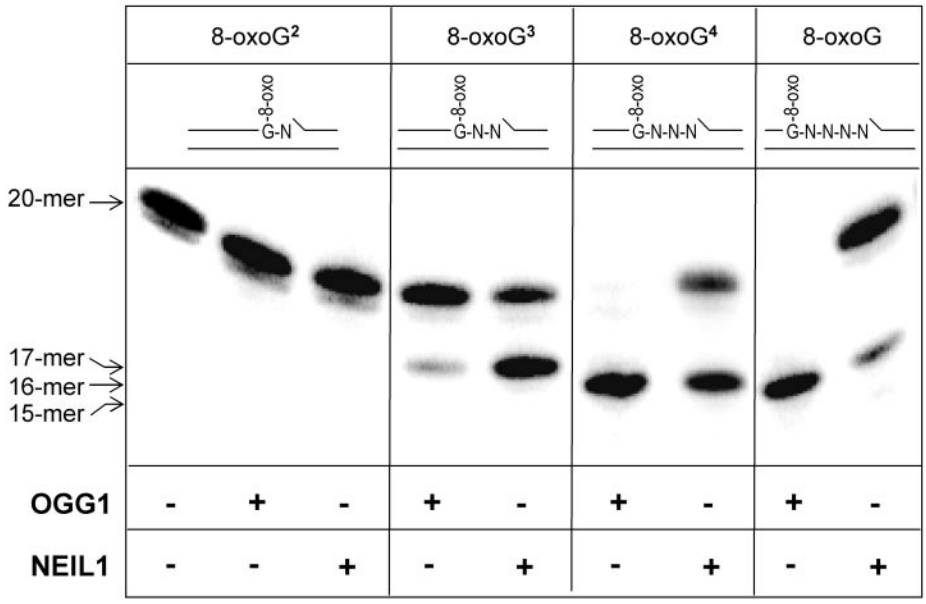

Figure 3. Comparison of the excision of 8-oxoG located near a DNA single-strand break by OGG1 and NEIL1. Oligonucleotide substrates (0.3 pmol), shown at the top of each panel, were incubated with either OGG1 (2.5 pmol) or NEIL1 (2.3 pmol) for 20 min at 37°C before treatment with 5% piperidine. The samples were subsequently dried and resuspended in formamide loading dye and an aliquot analysed by 20% denaturing PAGE and phosphorimaging. The first lane contains a size marker for untreated oligonucleotide substrate.

Table 1. Kinetic constants for excision of 8-oxoguanine from oligonucleotide substrates by OGG1 and NEIL1

Enzyme	Substrate	K_m (nM)	$k_{cat} \times 10^3$ (min^{-1})	$K_m/k_{cat} \times 10^3$ (min^{-1} nM^{-1})	Reference
NEIL1	5OHU:G	12.8	65.8	5.2	Dou *et al.* (2003) (19)
NEIL1	5OHU:G	5.0	90.5	18.0	This study
NEIL1	8oxoG^3:C	7.0	3.9	0.5	This study
NEIL1	8oxoG^4:C	13.8	2.8	0.2	This study
OGG1	8oxoG:C	23.0	34	1.5	Asagoshi *et al.* (2000) (18)
OGG1	8oxoG^3:C	51.8	3.6	0.07	This study
OGG1	8oxoG^4:C	41.3	45.4	1.1	This study

OGG1 and NEIL1 were incubated with increasing concentrations of the relevant oligonucleotide substrate for 10 min at 37°C prior to the addition of 5% piperidine. The samples were dried and subsequently resuspended in formamide loading dye and an aliquot was analysed by 20% denaturing polyacrylamide gel electrophoresis and phosphorimaging. Kinetic parameters were determined using Lineweaver–Burke plots and shown are data from our study in comparison to that already published.

although in comparison with an efficient substrate for NEIL1 (5-OHU), the turnover is at least 16-fold lower from both our estimates and those previously reported (19) but our data also indicate that the efficiency of excision of 5-OHU located near a SSB by NEIL1 (Figure 2) is relatively unaltered. Using an electrophoretic gel mobility shift assay (EMSA), we observed that NEIL1 has an ~5- to 10-fold higher binding affinity for 8-oxoG^3 than OGG1 indicating that NEIL1 is an efficient DNA glycosylase for this substrate (Figure 4).

Substrate specificity of NEIL1 against 8-oxoG located near a SSB

After we have demonstrated that NEIL1 is active against 8-oxoG^3, we further examined the requirement of NEIL1 for this particular substrate structure. First, we analysed the effect of the phosphorylation status of the DNA SSB on NEIL1 and OGG1 activity. We found that OGG1 activity was unaltered irrespective of whether the SSB was unphosphorylated, 3′- or 5′-phosphorylated in that very little excision was observed (Figure 5). However, NEIL1 activity was sensitive to the presence of a phosphate residue on either the 5′ or 3′ end of the SSB, with an ~3-fold reduction in activity observed in comparison with an unphosphorylated SSB (Figure 5). We also tested the activity of NEIL1 for 8-oxoG^3 located in single-stranded DNA, in a duplex substrate lacking the downstream strand, a nicked or gapped duplex substrate, and finally, 8-oxoG located in the centre of an oligonucleotide. We found that the excision activity of NEIL1 is very specific for 8-oxoG^3 in a nick-containing duplex DNA (Figure 6, substrate 3). The activity of NEIL1 on this substrate was ~2-fold greater than the activity on a gap-containing substrate (Figure 6, substrate, 4). If the DNA is single stranded (Figure 6, substrate 1), lacking the 3′-DNA strand (Figure 6, substrate 2) or the 8-oxoG is located within the centre of the substrate (Figure 6, substrate 5), then minimal or no activity is observed at the concentrations tested. Furthermore, we examined whether NEIL1 is involved in the excision of 8-oxoG using a splay

arm substrate modelling of the structure that is generated during DNA transcription or replication. We constructed a series of substrates where 8-oxoG was moved from the centre of the fork to positions 1–4 nucleotides 5′ away from the fork, although the cleavage product remains the same length (Figure 7). Interestingly, we observed that NEIL1 is not active against 8-oxoG within a splay arm structure. However, OGG1 is efficiently able to excise 8-oxoG from all substrates used (Figure 7).

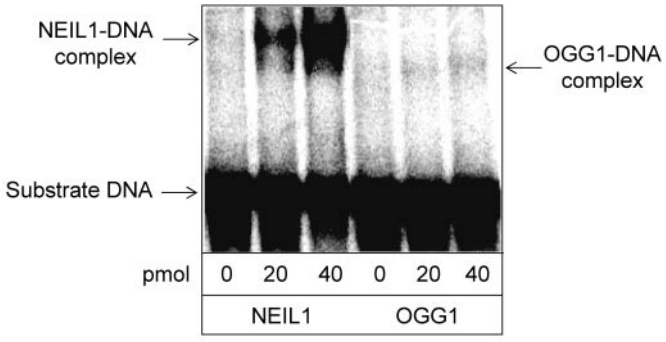

Figure 4. NEIL1 has a higher affinity for 8-oxoG[3] substrate. An oligonucleotide substrate (0.3 pmol) was incubated with 0, 20 and 40 pmol of either OGG1 or NEIL1 on ice for 15 min before separation by 12% non-denaturing gel electrophoresis at 4°C. The gel was dried and exposed to storage phosphor screens at 4°C before analysis by phosphorimaging.

DISCUSSION

ROS produced by oxidative metabolism or through ionizing radiation exposure may generate oxidative DNA damage located in close proximity to a SSB. OGG1 and NTH1 are the major DNA glycosylases that excise 8-oxoG and oxidized pyrimidines from DNA in human cells (6–9). Recently, the NEIL glycosylases, with a similar substrate specificity, have been isolated (12,13) although the question arises why we need these additional DNA glycosylases that have an overlapping substrate specificity to NTH1 and OGG1. The most obvious explanation is that the NEIL glycosylases provide a back-up repair system, however the observation that NEIL1 knock-down cells are sensitive to DNA damaging agents including low levels of γ-irradiation in the presence of fully active NTH1 and OGG1, challenged this theory (17). In this

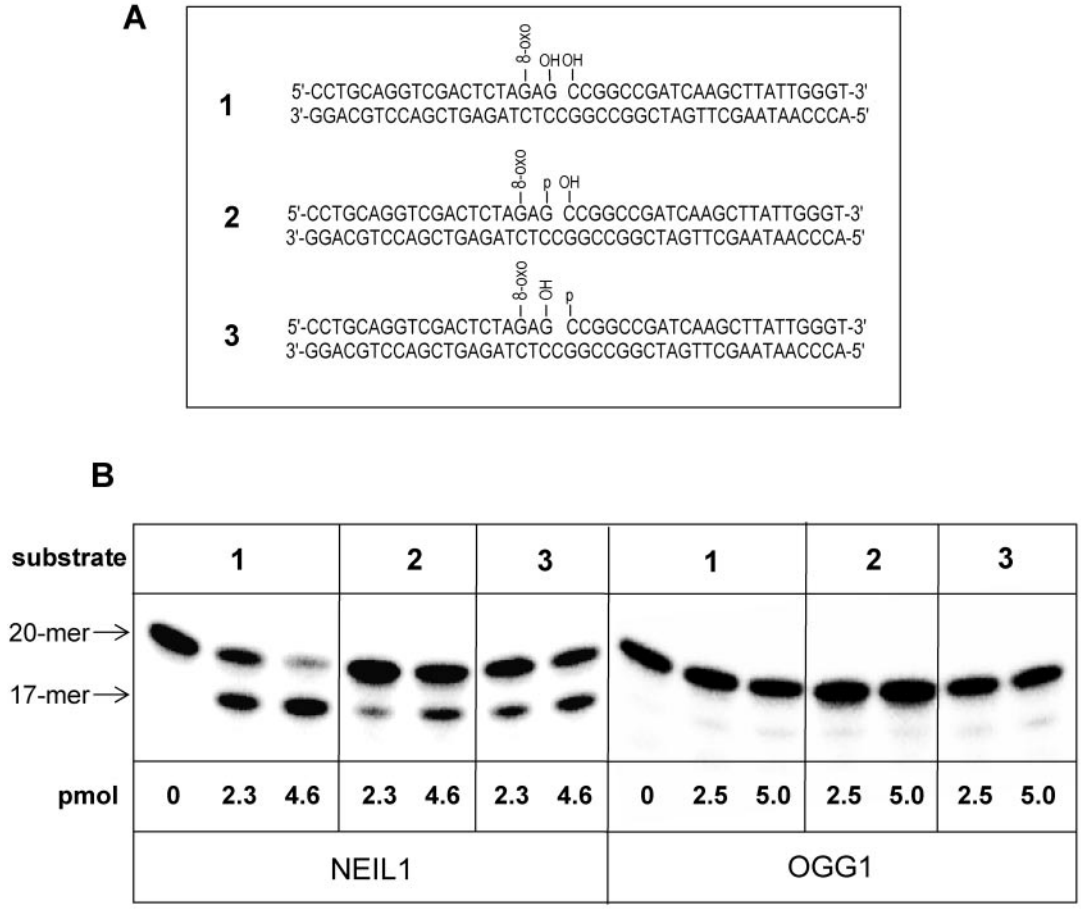

Figure 5. Effects of DNA single-strand break status on the excision of 8-oxoG[3] substrate by NEIL1 and OGG1. (**A**) Oligonucleotide substrates (0.3 pmol) were incubated with NEIL1 (2.3 or 4.6 pmol) or OGG1 (2.5 or 5 pmol) for 20 min at 37°C before treatment with 5% piperidine. The samples were subsequently dried and resuspended in formamide loading dye. (**B**) An aliquot was analysed by 20% denaturing PAGE and phosphorimaging. The first lane of each panel contains a size marker for untreated oligonucleotide substrate.

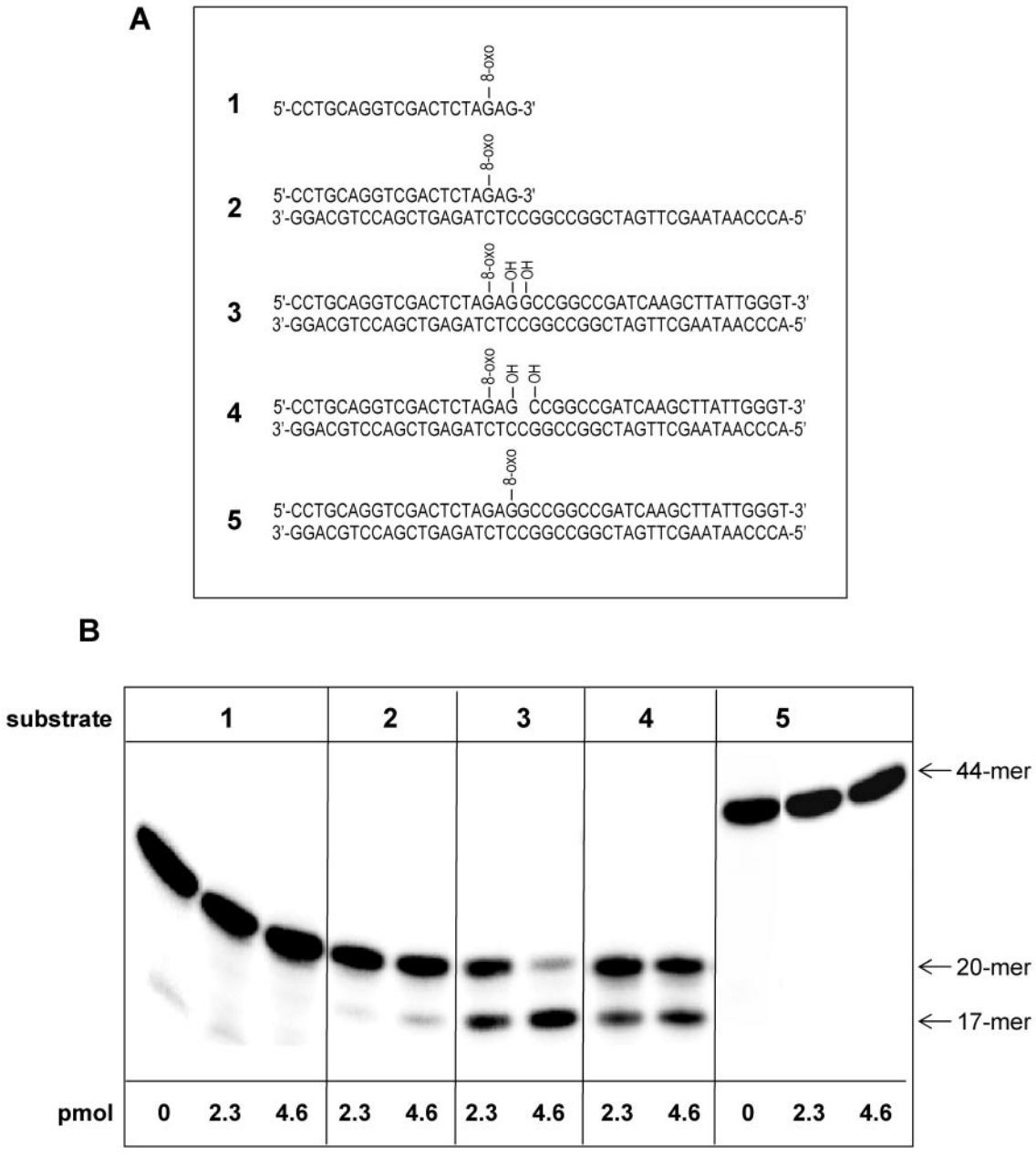

Figure 6. Structural requirements for 8-oxoG[3] incision by NEIL1. Oligonucleotide substrates (**A**) were incubated with NEIL1 (2.3 or 4.6 pmol) for 20 min at 37°C before treatment with 5% piperidine. The samples were subsequently dried and resuspended in formamide loading dye. (**B**) An aliquot was analysed by 20% denaturing PAGE and phosphorimaging. The first and last panels contain a size marker for untreated oligonucleotide substrate.

study, we demonstrated that NEIL1 has a unique ability to operate on oxidative DNA lesions located in close proximity to the 3′ end of a SSB where both NTH1 and OGG1 have reduced activity. However, we did not observe any activity of NEIL1 on hypoxanthine, a substrate for *N*-methylpurine DNA glycosylase, in close proximity to a DNA SSB (data not shown) and this indicates that the back-up repair activity of NEIL1 is specific for oxidative base damage excised by the major DNA glycosylases OGG1 and NTH1. Furthermore, none of the DNA glycosylases tested can excise 8-oxoG or 5-OHU at the second position from the 3′ end of a SSB and our results also suggest that APE1 is unable to excise these lesions through its exonuclease activity (data not shown), and further studies are underway to resolve the mechanism of repair of such lesions.

The observed activity of NEIL1 against 8-oxoG and 5-OHU located near a DNA SSB may help to explain the apparent sensitivity of NEIL1 knockdown cells to γ-irradiation (17), since γ-irradiation is known to induce lesions containing a combination of DNA strand breaks and oxidized bases, including 8-oxoG and 5-OHU (20). However, these lesions may not be the only substrates specific for NEIL1 that may help to explain this apparent increased sensitivity. Indeed, it has recently been demonstrated that NEIL1 is uniquely involved

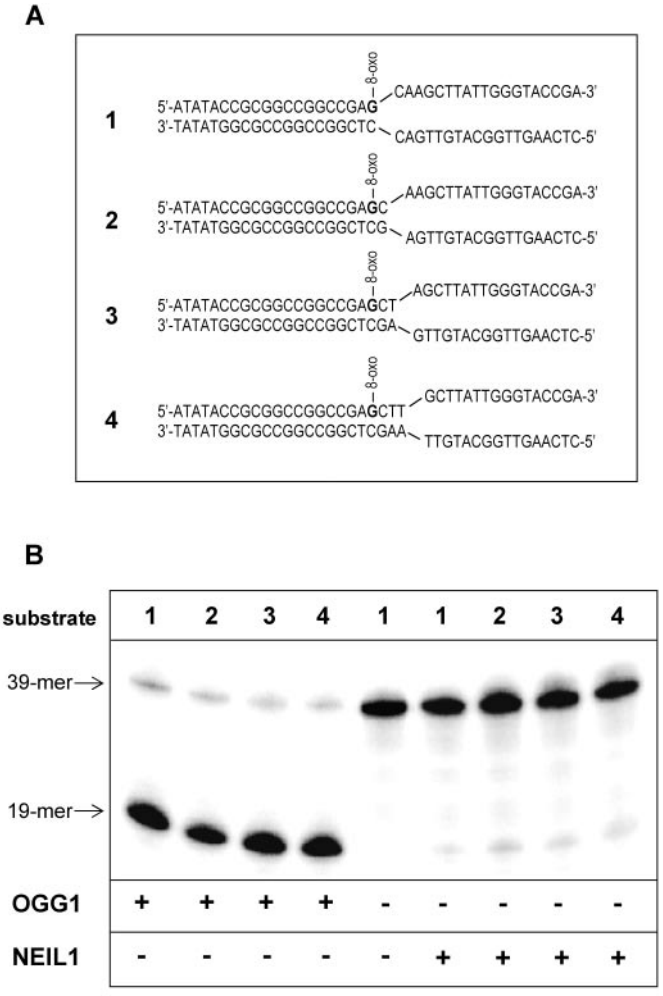

Figure 7. OGG1 and not NEIL1 is active on 8-oxoG within a splay arm structure. (**A**) Oligonucleotide substrates (0.3 pmol) containing 8-oxoG located 1–4 nt from the fork of a splay arm substrate were generated and incubated with either OGG1 (2.5 pmol) or NEIL1 (2.3 pmol) for 20 min at 37°C before treatment with 5% piperidine. (**B**) The samples were subsequently dried and resuspended in formamide loading dye and an aliquot analysed by 20% denaturing PAGE and phosphorimaging. The centre lane contains a size marker for untreated oligonucleotide substrate.

in the repair of oxidized bases in DNA bubble structures (19) and furthermore NEIL1 can excise Fapy A and the 5*S*,6*R* stereoisomer of thymine glycol that are resistant to OGG1 and NTH1 cleavage (17). We also examined the excision of 8-oxoG within a splay arm structure and found that OGG1 but not NEIL1 was able to efficiently excise 8-oxoG located 1–4 nt from the fork, indicating that OGG1, rather than NEIL1, may drive the repair of 8-oxoG during DNA transcription or replication.

Such striking differences in substrate specificity between OGG1, NTH1 and NEIL1 may have a structural basis. The crystal structure of OGG1 complexed with damaged DNA is known (21), although the structure of the NEIL1–DNA complex has not yet been solved but NEIL1 is homologous to Fpg and Nei, and closely resembles these proteins in its uncomplexed form. The crystal structure of human NEIL1 reveals a zinc-less finger motif required for DNA glycosylase activity (22), and the structure of the complex can reasonably be approximated by the complexes of Fpg (23) and Nei (24). In both OGG1 and Fpg/Nei, the second phosphate 3′ to the lesion coordinates catalytically important, highly conserved residues (Lys-249 and Lys-56, respectively), explaining the lack of activity of both OGG1 and NEIL1 towards the 8-oxoG[2] substrate, in which this phosphate is uncoupled from the damaged nucleotide by the nick. In contrast, the third phosphate 3′ to the lesion makes no important contacts with the enzymes of the Fpg/Nei family, whereas in OGG1 it contacts an absolutely conserved Gly-245 residue found in the loop between two helixes comprising a helix–hairpin–helix motif, which forms a significant part of the enzyme active site. Thus, introducing a break at this phosphate is probably detrimental for OGG1 but can be partially tolerated by NEIL1. Nevertheless, NEIL1 still prefers double-stranded DNA since its activity is noticeably reduced on single-stranded substrates or on substrates containing a single-nucleotide gap and it is more active on the substrate containing an unphosphorylated DNA strand break.

The crystal structure of covalently trapped bacterial endonuclease III with DNA has also been resolved (25). This reveals important contacts with the second and third phosphate 3′ to the lesion that may explain the lack of activity of NTH1 when a SSB is introduced at these sites. However, we also observed little excision activity of human NTH1 when a SSB is introduced at the fourth and fifth nucleotides 3′ to the lesion. This demonstrates that the human homologue of endonuclease III may contact the DNA at further sites 3′ to the lesion and indeed DNase I footprinting showed protection of 5 nt on the 3′ side of a 5,6-dihydrouracil lesion (26).

In summary, our data describe a new function for the DNA glycosylase NEIL1 in the excision of oxidative DNA damage located in close proximity to a SSB.

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Conflict of interest statement. None declared.

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