Differential Specificity of Human and Escherichia coli Endonuclease III and VIII Homologues for Oxidative Base Lesions*

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In human cells, oxidative pyrimidine lesions are restored by the base excision repair pathway initiated by homologues of Endo III (hNTH1) and Endo VIII (hNEIL1 and hNEIL2). In this study we have quantitatively analyzed and compared their activity toward nine oxidative base lesions and an apurinic/apyrimidinic (AP) site using defined oligonucleotide substrates. hNTH1 and hNEIL1 but not hNEIL2 excised the two stereoisomers of thymine glycol (5R-Tg and 5S-Tg), but their isomer specificity was markedly different: the relative activity for 5R-Tg:5S-Tg was 1.3:1 for hNTH1 and 5.1:1 for hNEIL1. This was also the case for their Escherichia coli homologues: the relative activity for 5R-Tg:5S-Tg was 1:2.5 for Endo III and 3.2:1 for Endo VIII. Among other tested lesions for hNTH1, an AP site was a significantly better substrate than urea, 5-hydroxyuracil (hoU), and guanine-derived formamidopyrimidine (mFapyG), whereas for hNEIL1 these base lesions and an AP site were comparable substrates. In contrast, hNEIL2 recognized an AP site exclusively, and the activity for hoU and mFapyG was marginal. hNEIL1, hNEIL2, and Endo VIII but not hNTH1 and Endo III formed cross-links to examine, suggesting conservation of the -fold of the active site of the Endo VIII homologues. The profiles of the excision of the Tg isomers with HeLa and E. coli cell extracts closely resembled those of hNTH1 and Endo III, confirming their major contribution to the repair of Tg isomers in cells. However, detailed analysis of the cellular activity suggests that hNEIL1 has a significant role in the repair of 5S-Tg in human cells.

DNA carrying vital genetic information of cells constantly suffers from spontaneous deamination and depurination, alklylation, and oxidation (1–3). These reactions lead to modifications of the DNA backbone and bases, with the latter predominating. The resulting aberrant bases are potentially genotoxic because of the loss or alteration of base pairing information (4), and hence need to be restored by the cellular repair system (2, 3, 5). The major repair mechanism for such damage is the base excision repair (BER) pathway (6), which is conserved from bacteria to humans. In the first step of BER, DNA glycosylases with distinct damage specificities detect the aberrant base in the vast sea of normal bases and remove it from the DNA backbone, leaving an apurinic/apyrimidinic (AP) site. The resulting AP site is further processed and repaired by the subsequent action of AP endonuclease (Endo), DNA polymerase, and DNA ligase through the short patch or long patch BER pathway.

The initial search for DNA glycosylases involved in the repair of oxidatively damaged bases in Escherichia coli identified Endo III, Endo VIII, and formamidopyrimidine-DNA glycosylase (7, 8). The principal substrates of Endo III and Endo VIII are oxidative pyrimidine lesions. They exhibit redundant damage specificity and catalyze the hydrolysis of the N-glycosidic bond (N-glycosylase activity) and the subsequent incision of an AP site by AP lyase activity via β-elimination (Endo III) or β,δ-elimination (Endo VIII). The E. coli mutants deficient in both Endo III and Endo VIII are strong spontaneous mutators (9, 10) and hypersensitive to the agents that generate reactive oxygen species such as ionizing radiation and hydrogen peroxide (9, 11). The principal substrates of formamidopyrimidine-DNA glycosylase are oxidative purine lesions, and it exhibits N-glycosylase and β,δ-AP lyase activities. The E. coli mutants deficient in formamidopyrimidine-DNA glycosylase are not sensitive to ionizing radiation but exhibit a mild spontaneous mutator phenotype (12, 13). Interestingly, while showing distinct substrate specificity, Endo VIII and formamidopyrimidine-DNA glycosylase belong to the same structural family, the Endo VIII/formamidopyrimidine-DNA glycosylase superfamily (14, 15).

The mammalian Endo III homologue (NTH1) and a functional homologue of formamidopyrimidine-DNA glycosylase (OGG1) have been identified previously, and their functions in BER have been assessed using purified proteins (16–22), knockout mice (23–28), and x-ray crystallographic analysis (29, 30). It has recently been shown that mammals have Endo VIII homologues (31, 32); they are designated NEIL1, NEIL2, and NEIL3 (after Nei-like), demonstrating the conserved organization of DNA glycosylases involved in the repair of oxidatively damaged DNA.

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The abbreviations used are: BER, base excision repair; Endo, endonuclease; hNTH1, human Nth homologue; hNEIL1 and hNEIL2, human Nei-like 1 and 2; Tg, thymine glycol; hoU, 5-hydroxyuracil; hoC, 5-hydroxycytosine; RU, 5-formyluracil; hM, 5-hydroxymethyluracil; AP, apurinic/apyrimidinic site; 8-oxoG, 7,8-dihydro-8-oxoguanine; mFapyG, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine; Oxa, oxazine; BSA, bovine serum albumin.
damaged pyrimidine and purine lesions. Studies into the repair function of the mammalian Endo VIII homologues reveal that like Endo III and Endo VIII, NTH1 and NEIL1/NEIL2 exhibit, albeit not fully, redundant damage specificity and primarily recognize oxidative pyrimidine lesions (14, 31–37). However, their activities toward oxidized base lesions have been assessed using different substrates (oligonucleotides with different sequence contexts or calf thymus DNA) and assay methods (nicking assays of DNA and release assays of damaged bases), making the quantitative comparison of activity data rather difficult. In light of this fact, we measured and quantitatively compared the activity of human NTH1, NEIL1, and NEIL2 (hNTH1, hNEIL1, and hNEIL2) and that of their E. coli homologues (Endo III and Endo VIII) using common oligonucleotide substrates. We report here that hNTH1, hNEIL1, and hNEIL2 exhibit significantly different activities toward the stereoisomers of thymine glycol (Tg) and other oxidative base lesions, and that this is also the case for Endo III and Endo VIII. These results, together with those obtained from cell extracts, indicate that base lesions generated by reactive oxygen species can be differentially attenuated in keeping with their repair kinetics.

EXPERIMENTAL PROCEDURES

Oligonucleotide Substrates—The substrates used in this study are listed in Table I. 30TG5R and 30TG5S containing the diastereoisomers 5R-Tg and 5S-Tg, respectively, were synthesized using the corresponding phosphoramidite monomers as described previously (38, 39). Tg has two diastereoisomers with respect to the configurations at C5 and C6: two cis isomers, (5R,6S)-Tg and (5S,6R)-Tg; and two trans isomers, (5R,6R)-Tg and (5S,6S)-Tg. The pair of 5R cis-trans isomers is in equilibrium because of epimerization in aqueous solution (abundance ratio, 87:13), and so is the pair of 5S cis-trans isomers (5S,6S)-Tg:5R,6S)-Tg and (5S,6R)-Tg:5R,6R)-Tg. Accordingly, the pair of 5R cis-trans isomers are abbreviated as 5R-Tg, and the pair of 5S cis-trans isomers as 5S-Tg throughout this paper. 30UR containing a urea residue was prepared by mild alkaline treatment of 30TG5R and 30TG5S (18, 41). 25FU, 25HMU, and 25OG containing 5-formyluracil (mFapyG), and oxanine (Oxa), respectively, were prepared by DNA polymerase reactions with modified 2-deoxynucleoside 5’-triphosphates as reported previously (19, 43–45). The oligonucleotides containing the base lesions were 5’-end labeled with [γ-32P]ATP (110 Tbp/ mmol, Amersham Biosciences) and T4 polynucleotide kinase (New England BioLabs) and purified by a Sep-Pak cartridge (Waters). The labeled oligonucleotides were annealed to appropriate complementary strands and used for activity assays. Duplex substrates are expressed as the combination of an oligonucleotide containing the lesion and the base opposite it (e.g. 30TG5R/A) throughout the paper. For the preparation of 19AP/A, a duplex oligonucleotide containing uracil at the position of the AP site was treated with uracil-DNA glycosylase (New England BioLabs) and purified by a Sep-Pak cartridge (Waters). The labeled oligonucleotides were annealed to appropriate complementary strands and used for activity assays. Duplex substrates are expressed as the combination of an oligonucleotide containing the lesion and the base opposite it (e.g. 30TG5R/A) throughout the paper. For the preparation of 19AP/A, a duplex oligonucleotide containing uracil at the position of the AP site was treated with uracil-DNA glycosylase (New England BioLabs).

DNA Glycosylases—The purification of Endo III, Endo VIII, and NTH1 were reported previously (19, 43). The native form of hNTH1 and hNEIL2 proteins were purified as follows. Briefly, based on published sequences (31, 32), hNTH1 and hNEIL2 cDNAs were amplified by PCR using the following primers: hNTH1 forward primer (5’- ATTTATCGCTGCTGCCTCAGT-3’), hNTH1 reverse primer (5’- GGCCAGCTGGCGATGCTGCTG-3’), hNEIL1 forward primer (5’- ATTTATCGCTGCTGCCTCAGT-3’), hNEIL1 reverse primer (5’- GGCCAGCTGGCGATGCTGCTG-3’), hNEIL2 forward primer (5’- ATTTATCGCTGCTGCCTCAGT-3’), hNEIL2 reverse primer (5’- GGCCAGCTGGCGATGCTGCTG-3’). The purified proteins were dialyzed in 20 mM Tris-HCl, pH 8.0, 75 mM NaCl, 10% glycerol, and 1 mM DTT, before being stored at −80 °C.

FIG. 1. Characterization of purified hNEIL1 and hNEIL2 proteins by SDS-PAGE and NaBH4 trapping. A, purified hNTH1, hNEIL1, and hNEIL2, and Endo VIII proteins (about 1 μg) were separated by 10% SDS-PAGE, and the gel was stained by Coomassie Brilliant Blue. The leftmost lane shows molecular mass markers (Daiichi Kagaku) with their sizes (kDa) indicated on the left. B, Endo VIII, hNTH1, and hNEIL2 were incubated with 19AP/A (19AP was 5’-end 32P-labeled) in the presence of 50 mM NaBH4 at 37 °C for 30 min. After incubation, the trapped complex and free DNA were separated by 10% SDS-PAGE. The autoradiogram of the gel is shown.
from the human liver cDNA library (Nippon Gene) using the polymerase chain reaction. The amplified DNA fragments were ligated into the NdeI/XhoI site of pET-22b (Novagen). The recombinant plasmids for hNEIL1 and hNEIL2 were designated phNEIL1 and phNEIL2, respectively.

E. coli BL21-CodonPlus (DE3)-RIL (Stratagene) was transformed with phNEIL1 or phNEIL2. The original sequence of the inserts was confirmed by sequencing phNEIL1 and phNEIL2 isolated from the host cell.

E. coli BL21-CodonPlus (DE3)-RIL harboring phNEIL1 or phNEIL2 was grown in LB media containing chloramphenicol (50 μg/ml) and ampicillin (50 μg/ml) at 37 °C until A600 reached 0.6. After the addition of isopropyl-β-D-thiogalactopyranoside (final concentration, 1 mM), the cell culture was continued at 30 °C for 3 h. The following procedures were performed at 4 °C or on ice. Harvested cells were disrupted by sonication. The cell lysate was centrifuged, and proteins in the supernatant were collected by ammonium sulfate precipitation (60% saturation). The hNEIL1 protein was purified by SP Sepharose CL-4B, MonoS, and Superdex 75 XK16/50 columns (all from Amersham Biosciences). The hNEIL2 protein was purified by SP Sepharose CL-4B (two cycles) and Superdex 75 XK16/50 (two cycles) columns. The pooled fraction containing hNEIL1 or hNEIL2 was dialyzed against 20 mM Hepes-KOH (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 50% glycerol, and stored at −80 °C (hNEIL1) or −20 °C (hNEIL2). The protein concentration was determined with the BCA protein assay kit (Pierce) using BSA as a standard.

Cell Extracts—Cell extracts were prepared on ice or at 4 °C. The HeLa cell extract was prepared from confluent cells. The cell pellet was suspended in three volumes of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 50% glycerol, and stored at −80 °C (hNEIL1) or −20 °C (hNEIL2). The protein concentration was determined with the BCA protein assay kit (Pierce) using BSA as a standard.

Fig. 2. PAGE analysis of the reaction products formed by incubation of oligonucleotide substrates containing 5'R-Tg and 5'S-Tg with Endo III and Endo VIII homologues. 30TG5R/A and 30TG5S/A (both 5 μm, the Tg strand was 5'-end 32P-labeled) containing 5'R-Tg and 5'S-Tg, respectively, were incubated with different amounts of the indicated enzymes at 37 °C for 30 min. Products were separated by 16% denaturing PAGE. The autoradiograms of the gels are shown. The amounts of enzyme used in the reactions were as follows (from left to right lanes for both 5'R-Tg and 5'S-Tg): 0, 0.25, 0.5, 1, and 2 ng for hNTH1; 0, 1, 2, 5, and 10 ng for hNEIL1; 0, 2.5, 5, 10, and 20 ng for hNEIL2; 0, 0.025, 0.05, 0.1, and 0.2 ng for Endo III; and 0, 0.5, 1, 2, and 4 ng for Endo VIII.

Fig. 3. Differential activities of Endo III and Endo VIII homologues for the 5'R-Tg and 5'S-Tg isomers. The percentage of nicked products was determined by the PAGE analysis as shown in Fig. 2, and is plotted against the amount of enzyme used for the assay (average of two experiments). Symbols: ○, 5'R-Tg; △, 5'S-Tg. The enzyme used is indicated above each panel.
n methylmethanesulfonyl fluoride, 1 \mu g/ml leupeptin, and 1 \mu g/ml pepstatin. The cells were disrupted with 30 strokes of a tight-fitting Dounce homogenizer and centrifuged at 100,000 × g for 30 min. The proteins in the supernatant were collected by ammonium sulfate precipitation against the same buffer, and used for activity assays. The protein concentration was determined sonication. The cell lysate was centrifuged, and the supernatant was dialyzed with the BCA protein assay kit.

The activity was calculated as [nicked substratel/enzyme/min, where square brackets denote the molar concentration.

| Enzyme | 5R-Tg | 5S-Tg | fU | hamU | Urea | hsoU | hsoC | 8-OxoG | mFapyG | AP |
|--------|-------|-------|----|------|------|------|------|--------|--------|----|
| hNTH1  | 1.4 × 10^{-2} | 1.1 × 10^{-3} | ND* | ND  | 1.1 × 10^{-2} | 4.9 × 10^{-3} | 1.7 × 10^{-3} | ND  | 6.8 × 10^{-3} | 2.3 × 10^{-2} |
| hNEIL1 | 5.1 × 10^{-3} | 3.3 × 10^{-3} | 3.5 × 10^{-4} | <1 × 10^{-5} | 4.2 × 10^{-3} | 3.8 × 10^{-3} | 5.9 × 10^{-4} | 3.8 × 10^{-4} | 4.4 × 10^{-3} | 3.5 × 10^{-3} |
| hNEIL2 | <1 × 10^{-5} | <1 × 10^{-5} | <1 × 10^{-5} | <1 × 10^{-5} | <1 × 10^{-5} | 5.1 × 10^{-5} | <1 × 10^{-1} | 2.7 × 10^{-5} | 2.7 × 10^{-3} |
| Endo III | 1.9 × 10^{-1} | 4.7 × 10^{-1} | ND  | ND  | 1.8 × 10^{-1} | 5.0 × 10^{-4} | 3.5 × 10^{-2} | ND  | 8.8 × 10^{-3} | 1.1 |
| Endo VIII | 3.0 × 10^{-2} | 9.3 × 10^{-3} | ND  | ND  | 2.0 × 10^{-2} | 1.6 × 10^{-4} | 1.7 × 10^{-3} | ND  | 3.6 × 10^{-4} | 1.6 × 10^{-2} |

* ND, activity was not determined.

**Fig. 4. Differential activities of Endo III and Endo VIII homologues for oxidative base lesions and AP sites.** The substrates (all 50 fmol) were incubated — Activity Assays — with the BCA protein assay kit.

**TABLE II**

**Activity of Endo III and Endo VIII homologues for oxidative base lesions and AP sites.** The substrates (all 50 fmol) were incubated — Activity Assays — with the BCA protein assay kit.

**RESULTS**

**Purification of hNEIL1 and hNEIL2** — The hNEIL1 and hNEIL2 proteins were overexpressed in E. coli and purified by several chromatographic steps. SDS-PAGE analysis of the purified hNEIL1 protein showed a single band with the expected molecular mass 43.6 kDa (Fig. 1A) (31, 32). The purified hNEIL2 protein (36.7 kDa) also showed a single band (Fig. 1A), but its mobility was comparable with that of a 42-4 kDa marker (aldolase). The unusual mobility of the hNEIL2 protein in SDS-PAGE agrees with the previous report (33). No bands were evident that indicated the contamination of Endo III or Endo VIII from the E. coli host. hNEIL1 and hNEIL2 were incubated with 19AP/A containing an AP site in the presence of NaBH₄ and the trapped reaction intermediate (a Schiff base formed between DNA and enzyme) was analyzed by SDS-PAGE (Fig. 1B). hNEIL1 and hNEIL2 gave rise to a single

**Cross-link Reactions with 25OXA** — The duplex of 25OXA/C (10 fmol) was incubated with hNEIL1 or hNEIL2 (200 ng) in the activity assay buffer described above (10 ml) at 37 °C for up to 1 h. The sample was mixed with SDS-loading buffer, heated, and separated by 10% SDS-PAGE. Autoradiography and quantitation of the radioactivity were performed as described above.

**Differential Damage Specificity of hNTH1, hNEIL1, and hNEIL2**

Data are derived from the linear part of plots in Figs. 3 (5R- and 5S-Tg) and 4 (hoU, hoC, mFapyG, and AP), and the results not shown (urea, fU, hamU, and 8-oxoG). The amount of proteins was varied depending on the activity of enzyme used is indicated by PAGE analysis. The percentage of nicked products is plotted against the enzyme used for the assay (av.

**Symbols**

- AP site
- hNEIL1
- hoC
- hNEIL2
- mFapyG

**Cross-link Reactions with 25OXA** — The duplex of 25OXA/C (10 fmol) was incubated with hNEIL1 or hNEIL2 (200 ng) in the activity assay buffer described above (10 ml) at 37 °C for up to 1 h. The sample was mixed with SDS-loading buffer, heated, and separated by 10% SDS-PAGE.
specificity of human and *E. coli* indicated against the lesions. The enzyme used is base lesions in Table II were standardized for each enzyme, the activities for selected homologues (Endo III and Endo VIII) were incubated with 30TG5R/A and 30TG5SS/A containing 5R-Tg and 5S-Tg, respectively, and the products were analyzed by denaturing PAGE (Fig. 2). Fig. 3 shows plots of the amount of nicked products (average of two experiments) against that of enzyme used for the assay, which was varied depending on the activity. *hNTH1* and *hNEIL1* recognized both 5R-Tg and 5S-Tg isomers, but their specificity for the isomers differed significantly. *hNTH1* excised 5R-Tg much more preferentially compared with 5S-Tg (Fig. 3A), whereas *hNEIL1* excised 5R-Tg only slightly better than 5S-Tg (Fig. 3B). When 5R-Tg and 5S-Tg in 30TG5R and 30TG5S, respectively, were converted to urea residues by mild alkaline treatment, *hNTH1* and *hNEIL1* exhibited the same activity toward urea residues derived from the two Tg isomers (data not shown). These results confirmed that the differential specificities of *hNTH1* and *hNEIL1* toward the Tg isomers originate from the distinct configurations at C-5 and C-6 of the pyrimidine ring. The activity of *hNEIL2* for the Tg isomers was below the detection limit (Fig. 3C). From the slope of the essentially linear part of the plot in Fig. 3, the activity for the two Tg isomers was calculated as [nicked substrate]/[enzyme]/min, where square brackets denote the molar concentration, and is summarized in Table II. According to the data in Table II, the specificity ratio toward 5R-Tg versus 5S-Tg is 13:1 for *hNTH1* (i.e. $1.4 \times 10^{-2}$ min$^{-1}$ versus $1.1 \times 10^{-3}$ min$^{-1}$) and 1.5:1 for *hNEIL1* (i.e. $5.1 \times 10^{-3}$ min$^{-1}$ versus $3.3 \times 10^{-3}$ min$^{-1}$), demonstrating marked differences in the isomer specificity between *hNTH1* and *hNEIL1*. It can also be deduced from the activity data (Table II) that for 5R-Tg, *hNTH1* exhibits a higher turnover rate than *hNEIL1* (*hNTH1*: *hNEIL1* = 2.7:1), whereas for 5S-Tg, *hNEIL1* exhibits a higher turnover rate than *hNTH1* (*hNTH1*: *hNEIL1* = 1:3). These results are in contrast to those reported recently for mouse NTH1 (mNTH1) and NEIL1 (mNEIL1) (36), although the isomer specificities of mNTH1 and mNEIL1 for the two Tg isomers are similar to those observed for *hNTH1* and *hNEIL1* in this study. The ratio of the turnover rates for 5R-Tg (estimated from the reported data) is mNTH1: mNEIL1 = 1.29, and that for 5S-Tg is 1.57, indicating that mNEIL1 is an extremely efficient enzyme as compared with mNTH1 for both Tg isomers, which was not the case for *hNEIL1* (see above).

The activity of *E. coli* Endo III and Endo VIII for the two Tg isomers was determined in a similar manner (Figs. 2 and 3). Like *hNTH1* and *hNEIL1*, Endo III and Endo VIII exhibited

![Fig. 5. Comparison of the damage specificity of human and *E. coli* Endo III and Endo VIII homologues. For each enzyme, the activities for selected base lesions in Table II were standardized to that for an AP site, and are plotted against the lesions. The enzyme used is indicated above each panel.](image1)

![Fig. 6. Differential excision capacities of HeLa and *E. coli* cell extracts for the 5R-Tg and 5S-Tg isomers. 30TG5R/A and 30TG5SS/A (both 5 nM) containing 5R-Tg and 5S-Tg, respectively, were incubated with different amounts of extracts from HeLa and *E. coli* cells at 37°C for 30 (HeLa) or 10 min (*E. coli*). Products formed with the HeLa (panel A) and *E. coli* (panel B) extracts were separated by 16% denaturing PAGE. The bands of β-elimination and β,δ-elimination products are indicated with β and δ, respectively. The amounts of HeLa and *E. coli* cell extracts used in the reaction were 0, 0.5, 1, 2, and 4 μg (from left to right lanes for both 5R-Tg and 5S-Tg). The percentage of nicked products was determined by the PAGE analysis as described above, and is plotted against the amount of the cell extract used for the assay. Panels C and D show the results with the HeLa and *E. coli* cell extracts, respectively. Symbols: ●, 5R-Tg; ▲, 5S-Tg.](image2)
Differential Damage Specificity of hNTH1, hNEIL1, and hNEIL2

significantly different specificities for the two isomers. However, Endo III excised 5S-Tg better than 5R-Tg (Fig. 3D), and the specificity ratio toward 5R-Tg versus 5S-Tg was 1.25 (i.e. $1.9 \times 10^{-1}$ versus $4.7 \times 10^{-1}$ min$^{-1}$, Table II). Thus, despite being homologues, hNTH1 and Endo III have an opposite preference for the Tg isomers. Endo VIII preferentially excised 5R-Tg as compared with 5S-Tg (Fig. 3E), and the specificity ratio toward 5R-Tg versus 5S-Tg was 3.2:1 (i.e. $3.0 \times 10^{-2}$ min$^{-1}$ versus $9.3 \times 10^{-3}$ min$^{-1}$, Table II). Although hNHE1 has a slight preference of 5R-Tg over 5S-Tg, the difference in the isomer specificity of Endo VIII (3.2-fold) is greater than that of hNHE1 (1.5-fold). It is likely from the activity data (Table II) that Endo III exhibits higher turnover rates than Endo VIII for both 5R-Tg (6.3-fold) and 5S-Tg (51-fold).

Activity of Purified Enzymes for Other Oxidative Base Lesions—hNTH1, hNEIL1, hNEIL2, Endo III, and Endo VIII were incubated with the substrates containing hoU (25HOUC/G), hoC (25HOC/G), mFapyG (34FP/C), an AP site (19AP/A), a urea residue (19UR/A), fu (25FUUA), hmU (25HMUA), and 8-oxoG (25OG/C), and products were analyzed by denaturing PAGE (data not shown). Fig. 4 shows typical plots of the damage specificity of hNTH1, hNEIL1, and hNEIL2 (and data not shown), implying a common architecture of the active site pocket in human and E. coli enzymes.

The profiles of the excision of the two Tg isomers with the HeLa cell extract (Fig. 6A) were similar to that with hNTH1 (Fig. 3A). In contrast, the E. coli cell extract preferentially excised 5S-Tg as compared with 5R-Tg. The profiles of the excision of the two Tg isomers with the E. coli cell extract (Fig. 6D) were similar to that with Endo VIII (Fig. 3D). The “Discussion” provides more detailed analysis of the cellular activity for the Tg isomers.

**Table III**

| Cell     | Enzyme | $P_{SS/5R}$ | $I_{SS}$ | $P_{SS}$ | $I_{SS}$ |
|----------|--------|-------------|---------|---------|---------|
| Mammal   | NTH1   | 0.079       | 7.1–7.5 | 52–70   | 90–95   |
| E. coli  | NEIL1  | 0.65        | 3.2–6.5 | 30–48   | 5–10    |
| E. coli  | Endo III | 2.5       | 220–240 | 99      | 90–95   |
| E. coli  | Endo VIII | 0.31    | 1.6–3.1 | 1       | 5–10    |

The activity ratio of purified enzymes for 5S- and 5R-Tg isomers (calculated from the data in Table II).

$P_{SS} = P_{SS}/P_{5R}$ for individual enzymes.

$P_{SS} = I_{SS}/(sum$ of $I_{SS}$ values for NTH1 and NEIL1) $\times 100$ for the mammalian enzymes, and $P_{SS} = I_{SS}/(sum$ of $I_{SS}$ values for Endo III and Endo VIII) $\times 100$ for the E. coli enzymes.

The contribution of enzymes to the excision of 5R-Tg was estimated from the data with NTH1-knockout mice or E. coli nth mutants (see text).

**Fig. 7. Cross-link formation of hNHE1 and hNEIL2 with Oxa.** 25OXA/C (the Oxa strand was 5'-end $^{32}$P-labeled) was incubated with hNHE1 or hNEIL2 at 37 °C for 0, 10, 30, 45, and 60 min. The resulting cross-link complexes containing 25OXA and hNHE1 or hNEIL2 were separated from free 25OXA by 10% SDS-PAGE.
In this study we have shown that hNTH1, hNEIL1, and hNEIL2 exhibit quantitatively different specificities toward oxidatively damaged bases and AP sites. hNTH1 and hNEIL1 recognized both oxidatively damaged bases and AP sites, showing a redundant spectrum of damage recognition. However, the specificity toward the individual lesions differs significantly between hNTH1 and hNEIL1 (Fig. 5, A and B). This was also the case for their E. coli homologues (Endo III and Endo VIII, Fig. 5, D and E). In contrast, hNEIL2 recognized AP sites exclusively, and the excision efficiency for the oxidatively damaged bases (hoU and mFapyG) was low (Fig. 5C). The present results, together with those reported for mammalian NTH1 (16–19), NEIL1 (14, 31–37), and SMUG1 (43, 44), show an elaborate backup system of mammalian DNA glycosylases that work in the first step of BER for oxidatively damaged DNA. For example, at least two or three enzymes can participate in the repair of Tg and urea residues (NTH1 and NEIL1), hoU (SMUG1, NTH1, and NEIL1), and mFapyG and its unmethylated analogue (OGG1, NTH1, and NEIL1). This may partly explain the lack of overt phenotypes of NTH1-knockout mice (23, 24). The repair function of hNEIL2 in BER needs to be further assessed because it excises hoU and mFapyG with only limited efficiency (Fig. 5C). In addition, hNEIL1 and hNEIL2 excise oxidative base lesions in single-stranded and bubble DNA structures, suggesting their repair role during DNA replication and/or transcription (34, 37).

Interestingly, the two Tg isomers were excised from DNA with differential efficiencies by both HeLa and E. coli cell extracts, and the specificity for the isomers was opposite between HeLa and E. coli cells (Fig. 6, C and D). It is known that ionizing radiation generates the two Tg isomers at comparable rates (46). Thus, the present results indicate that the 5R-Tg isomer is preferentially removed from chromosomal DNA in irradiated human cells, whereas the 5S-Tg isomer is preferentially removed in irradiated E. coli cells. The 5R-Tg and 5S-Tg isomers exert a similar destabilizing effect on duplex DNA (39) and equally constitute strong blocks to DNA synthesis catalyzed by polymerase α (47). However, with polymerase η, translesion synthesis past 5R-Tg is more efficient than that past 5S-Tg (47). Thus, 5S-Tg is a more persistent as well as intense blocking lesion than 5R-Tg because of slow removal and translesion synthesis (at least for polymerase η), and possibly constitutes a burden for mammalian cells. It remains to be seen whether the two isomers have distinct effects on other biological processes such as DNA replication catalyzed by other replicative and TLS polymerases or transcription, and also whether the species-specific excision of a particular Tg isomer results in any distinct biological consequences.

The kinetic profiles of the excision of the Tg isomers with the HeLa (Fig. 6C) and E. coli (Fig. 6D) cell extracts closely resembled those of hNTH1 (Fig. 3A) and Endo III (Fig. 3D), respectively, suggesting their major contribution to the cellular activity to Tg. This is also consistent with the observation that the residual activity for 5R-Tg (prepared by chemical oxidation of T with KMnO$_4$ or OsO$_4$) in cells deficient in NTH1 (NTH1-knockout mouse) or Endo III (E. coli nth mutant) is 5–10% of the wild type cells (29, 23, 48) (and data not shown). Given that the residual activity for 5R-Tg is attributable to NEIL1 and Endo VIII in mammalian and E. coli cells, respectively, the contribution of the individual enzymes to the excision of 5S-Tg in cells (P$_{5S}$) can be estimated using their activity ratio for the 5S-Tg and 5R-Tg isomers (P$_{5S}/$P$_{5R}$) and observed cellular activity for the 5R-Tg isomer (P$_{5R}$). The estimated contribution of hNTH1 to the excision of 5S-Tg (P$_{5S}$) is 52–70% and that of hNEIL1 is 30–48% (Table III), indicating fairly active involvement of hNEIL1 in the repair of 5S-Tg in human cells. This is mainly because of the higher turnover rate of hNEIL1 for 5S-Tg (3-fold) than that of hNTH1. Similar calculations for the E. coli enzymes (P$_{5S}$ in Table III) show that Endo III contributes exclusively to the repair of 5S-Tg (99%) relative to Endo VIII (1%) in E. coli cells. The distinct contributions of hNEIL1 and Endo VIII to the repair of the 5S-Tg isomer agree semi-quantitatively with the presence of the β,ε-elimination product (the hallmark of hNEIL1) with the Hela cell extract and its absence (the hallmark of Endo VIII) with the E. coli cell extract (lanes for 5S-Tg in Fig. 6, A and B).

We have previously shown that Oxa, a major guanine lesion produced by the reaction with nitric oxide or nitrous acid (49, 50), forms cross-links with DNA-binding proteins such as histone, HMG protein, and DNA glycosylases (45). The reaction with histone and HMG proteins is very slow, occurring on a time scale of days, but that with DNA glycosylases is very rapid, occurring in less than an hour, and possibly involves the direct interaction of Oxa in DNA with the active site residue of DNA glycosylases (tentatively assigned to Lys or Arg). Interestingly, despite sharing activities for oxidative pyrimidine lesions, Endo VIII but not Endo III and hNTH1 are cross-linked to Oxa (45). Accordingly, Oxa is a simple but useful lesion to probe the architecture of the active site of DNA glycosylases. The present study has shown that hNEIL1 and hNEIL2 form cross-links to Oxa at rates comparable with that of Endo VIII (Fig. 7), although they exhibit no appreciable N-glycosylase activity toward Oxa. This result suggests that hNEIL1, hNEIL2, and Endo VIII share in common the -fold of the active site, which is distinct from that of Endo III and hNTH1. Similar to Endo VIII (51, 52), hNEIL1 and hNEIL2 are likely to have a capacity to accommodate certain types of purine lesions, but not all of them are excised. The determination of the three-dimensional structures of hNEIL1 and hNEIL2 and their comparison to those of Endo VIII (53), Endo III (54, 55), and hNTH1 will provide further insight into the mechanisms underlying the differential recognition of oxidatively damaged bases by these enzymes. Toward the end of the preparation of this manuscript, two papers showing the differential specificity of Endo III and Endo VIII homologues toward the Tg isomers became in press (56, 57).

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