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Identification and Characterization of a Novel Human DNA Glycosylase for Repair of Cytosine-derived Lesions*

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Two candidate human orthologs of *Escherichia coli* MutM/Nei were recently identified in the human genome database, and one of these, NEH1, was characterized earlier (Hazra, T. K., Izumi, T., Boldogh, I., Imhoff, B., Kow, Y. W., Jaruga, P., and Dizdaroglu, M. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 3523–3528). Here we report characterization of the second protein, originally named NEH2 and now renamed NEIL2 (Nei-like). The 37-kDa wild-type NEIL2 expressed in and purified from *E. coli* has DNA glycosylase/AP lyase activity, primarily for excising oxidative products of cytosine, with highest activity for 5-hydroxyuracil, one of the most abundant and mutagenic lesions induced by reactive oxygen species, and with lower activity for 5,6-dihydrouracil and 5-hydroxycytosine. It has negligible or undetectable activity with 8-oxoguanine, thymine glycol, 2-hydroxyadenine, hypoxanthine, and xanthine. NEIL2 is similar to NEIL1 in having N-terminal Pro as the active site. However, unlike NEIL1, its expression was independent of the cell cycle stage in fibroblasts, and its highest expression was observed in the testes and skeletal muscle. Despite the absence of a putative nuclear localization signal, NEIL2 was predominantly localized in the nucleus. These results suggest that NEIL2 is involved in global genome repair mainly for removing oxidative products of cytosine.

Reactive oxygen species (ROS),[1] generated both endogenously and exogenously, have been implicated in the etiology of a variety of pathophysiological states ranging from cancer to rheumatoid arthritis, cardiovascular diseases, and Alzheimer's disease and also in aging (1–3). ROS are critically genotoxic because their reaction with DNA produces a plethora of mutagenic and toxic base lesions as well as DNA strand breaks (4). Most of these lesions, except double strand breaks, are repaired primarily via the base excision repair (BER) pathway in a series of enzymatic steps initiated with the excision of oxidatively damaged bases by several DNA glycosylases. These enzymes have broad substrate range although with preference for specific substrates.

Until recently only two DNA glycosylases, NTH1 and OGG1, had been identified in mammals for repair of oxidized base lesions (5, 6). Both of these are of the *Escherichia coli* Nth type and utilize a specific, internal Lys residue as the active site nucleophile to carry out β-elimination after base removal. Consequently, DNA strand breaks containing 3′-phospho-α,β-unsaturated aldehyde groups are generated (7). However, *E. coli* possesses two other oxidized base-specific DNA glycosylases, namely MutM and Nei, which form a distinct class because they utilize N-terminal Pro as the active site to carry out βδ-elimination and generate 3′-phosphate at the DNA cleavage site (8, 9). We recently identified two orthologs of MutM/Nei in the human genome database and named them Nei homologs NEH1 or NEH2 (10). NEH1 has since been renamed NEIL1 (Nei-like) as suggested by the Human Genome Organization. We showed that NEIL1 functions as a DNA glycosylase/AP lyase with broad substrate specificity, behaves more like Nei in its substrate preference, and carries out βδ-elimination like MutM and Nei (10). In this communication, we show that NEIL2 (originally named NEH2) is also active as a DNA glycosylase/AP lyase with the N-terminal Pro as the active site for carrying out βδ-elimination on DNA abasic sites. However, NEIL2 is distinct from NEIL1 in that ROS-generated cytosine derivatives are its preferred substrates.

EXPERIMENTAL PROCEDURES

Human Genome Database Analysis and Molecular Cloning of NEIL2—Identification of two human cDNA clones of NEIL1 (NEH1) and NEIL2 (NEH2), along with their mouse homologs, in the National Center for Biotechnology Information (NCBI) and Celera Genomic Databases using the BLASTP program was described previously (10). The cDNA containing the complete coding sequence of human NEIL2, obtained from Research Genetics (accession no. BC013964), was inserted between the *Nde*I and *Xho*I sites of the pRSETB plasmid, and its identity was verified by sequence analysis. Predicting that the N-terminal Pro is the catalytic site (after cleavage of the initiator Met residue), we also constructed expression plasmids encoding the N-terminal His tag fusion of NEIL2 in which this Pro became an internal residue.

Purification of Recombinant NEIL2 from E. coli—Recombinant, wild-type (WT) NEIL2 was purified as described for NEIL1 in a series of chromatographic steps with some modification (10). Briefly, the enzymatically active fractions eluted from SP-Sepharose at 300 mM NaCl were chromatographed on a 1-ml HiTrap heparin column and eluted at 0.5 M NaCl. After chromatography on Mono S, the final step involved gel filtration in a 25-ml Superdex 75 column equilibrated in 25 mM Tris-HCl (pH 7.5), 10% glycerol (buffer A) containing 200 mM NaCl. The

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[1] The abbreviations used are: ROS, reactive oxygen species; AP, abasic; APE, AP endonuclease; DHU, 5,6-dihydrouracil; 5-OHC, 5-hydroxycytosine; 5-OHU, 5-hydroxyuracil; NEH, Nei homolog; Nei, endonuclease VIII; NEIL, Nei-like; Nth, endonuclease III; NTH1, Nth homolog 1; OGG1, 8-oxoguanine-DNA glycosylase I; WT, wild-type; BER, base excision repair; FITC, fluorescein 5-isothiocyanate; PI, propidium iodide; MT, MitoTracker Red.

active fractions were stored in 25 mM Tris-HCl (pH 7.5), 150 mM KCl, 50% glycerol at −20 °C. The final yield of the purified enzyme was about 2 mg/liter of culture.

His-tagged NEIL2 was purified from the extract of plasmid-bearing *E. coli* by affinity chromatography on nickel-nitriloacetic acid-agarose (Qiagen). After elution with 150 mM imidazole, the enzyme was dialyzed against 20 mM Tris-HCl (pH 7.5), 250 mM NaCl and digested with thrombin to remove the His tag sequence except for 3 terminal residues. The enzyme was finally purified by chromatography on Mono S as in the case of WT NEIL2.

NEIL2 is highly unstable and loses activity nearly completely after storage at −80 °C or after 15-min incubation at 37 °C. The purified enzyme is therefore never frozen, and the enzyme retains full activity when assayed in the presence of 1 M trimethylamine *N*-oxide, a natural osmolyte that is present in marine organisms and known to stabilize proteins (11, 12). The enzyme is maximally active in 50 mM KCl and at pH 8.0.

Assay of NEIL2 with Oligo Substrates—DNA strand cleavage at the site of the lesion by the intrinsic AP lyase activity of NEIL2 after excision was used for its assay using ^{32}P-labeled duplex oligo substrates containing various base lesions (10). A 51-mer oligo containing 5-hydroxyuracil (5-OHU) (Integrated DNA Technology, Coralville, IA) or 5,6-dihydrouracil (DHU) (Midland Certified Reagent Co., Midland, TX) in the sequence 5′-GCT TAG CTT GGA ATC GTA TCA TGT A*X*A CTC GTG TGC CGT GTA GAC CGT GCC-3′, where *X* is 5-OHU or DHU, and the complementary strand containing G or A opposite DHU or G, A, or T opposite 5-OHU were used. The duplex oligo, ^{32}P-labeled at the 5′ terminus of the lesion-containing strand, was incubated with NEIL2 at 37 °C for 15 min (unless stated otherwise) in a 15-μl reaction mixture containing 50 mM Hepes (pH 8.0), 50 mM KCl, 100 μg/ml bovine serum albumin, 1 M trimethylamine *N*-oxide. After the reaction was stopped with 80% formamide, 10 mM NaOH, cleaved oligos were separated by denaturing gel electrophoresis in 15% polyacrylamide containing 7 M urea in 90 mM Tris borate (pH 8.3), 2 mM EDTA, and the radioactivity was quantitated by PhosphorImager (Amersham Biosciences) analysis. NEIL2-cleaved products were identified by comparison with the reference products of human NTH1 and NEIL1.

Analysis of Trapped Complex of NEIL2—The abasic (AP) site-containing ^{32}P-labeled duplex oligo was incubated with enzymes in 15 μl of assay buffer in the presence of 25 mM $NaCNBH_3$ at 37 °C for 30 min, and the trapped complexes were separated by SDS-PAGE (12% polyacrylamide) as described previously (10).

Cell Synchronization and Analysis of Cell Cycle—Primary human diploid MRC5 fibroblasts were synchronized by serum starvation for 120 h and then stimulated to proliferate by adding fetal bovine serum (10%) to the Eagle's minimal essential medium. Aliquots of cells, harvested at various times, were used for cell cycle analysis and for preparing lysates for blot analysis of NEIL2 mRNA (10).

Determination of Subcellular Localization of NEIL2 Gene—The FLAG epitope tag was fused to the 3′ terminus of the NEIL2 cDNA as follows. Two oligonucleotides, 5′-A ATT C TC GACTACAAAGACGATGACGACAAG TAAC-3′ and 5′-T CG AGTTACTTGTCGTCATCGTCTTTG TAGTCGA G-3′, were annealed and inserted into the *Eco*RI-*Xho*I sites of pcDNA3.0Zeo(+) vector (Invitrogen), which was then used to clone the NEIL2 cDNA into its *Hin*dIII-*Eco*RI sites, in-frame with the FLAG tag. The NEIL2-FLAG plasmid (0.5 μg) was transiently transfected into mouse BALB/c 3T3 A31-1-1 fibroblasts (13) using LipofectAMINE 2000 (Invitrogen). After 24 h, the cells were fixed and treated with M3 anti-FLAG antibody conjugated with fluorescein 5-isothiocyanate (FITC) (Sigma) for 1 h. Then the cells were stained with propidium iodide (PI) or MitoTracker Red (MT) (Molecular Probes) according to the manufacturers' instructions and analyzed by confocal fluorescence microscopy (Zeiss LSM510) in the University of Texas Medical Branch Optical Imaging Laboratory. FITC was imaged using 488 nm excitation and 505–545 nm emission, while PI and MT were imaged using 543 nm excitation and 560 nm long pass emission.

RESULTS

Purification and Characterization of DNA Glycosylase Activity of Recombinant NEIL2—The overall identity between human NEIL2 and MutM and Nei is 32 and 27%, respectively (Pairwise BLAST analysis), and the key residues of the *E. coli* enzymes, in particular the N-terminal PE(L/G)P(E/L) motif, are completely conserved in NEIL2 (10). This suggests that, as in NEIL1, the N-terminal Pro of NEIL2 is the active site.

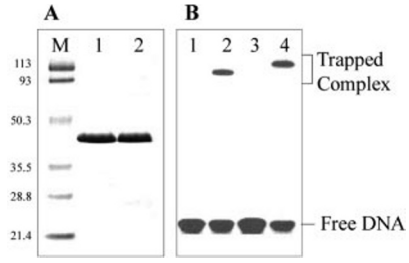

FIG. 1. *A*, purification of NEIL2. *Lane 1*, WT NEIL2; *lane 2*, NEIL2 with N-terminal tag. Bio-Rad protein markers (*M*) are shown on the *left*. *B*, trapping analysis of purified (5 ng) enzyme with 0.1 pmol of AP site-containing duplex oligo. *Lane 1*, no protein; *lanes 2–4*, WT, N terminus-tagged NEIL2, and NEIL1, respectively. The positions of free DNA and trapped complexes are indicated.

Furthermore, NEIL2 shares potential helix-2-turn-helix and zinc finger motifs with both MutM and Nei. We expressed WT NEIL2 and a fusion protein with an N-terminal His tag in which Pro-1 becomes an internal residue and purified these to apparent homogeneity (Fig. 1*A*). Surprisingly, 36.7-kDa NEIL2 migrated abnormally to the position of 42 kDa in SDS-PAGE. The N-terminal sequence of the WT protein (Pro-Glu-Gly-Pro-Leu-Val-Arg-Lys-Phe, as determined by automated Edman degradation) matched exactly with the predicted sequence (after cleavage of the initiator Met residue). The molecular weight of WT NEIL2 of 36,691, as determined by mass spectrometry analysis, was close to the predicted value of 36,695. Similarly the N-terminal sequence of the His-tagged fusion protein after thrombin cleavage (Gly-Ser-His-Met-Pro-Glu-Gly) also matched perfectly with the predicted sequence.

All DNA glycosylase/AP lyases, regardless of preference for the substrate base, form transient Schiff bases with free abasic sites in DNA which could be reduced with $NaCNBH_3$ (or $NaBH_4$) to form stable "trapped complexes" (14, 15). The MutM/Nei-type enzyme is inactivated if the N-terminal Pro, the active site nucleophile, is blocked or eliminated (10). Fig. 1*B* shows SDS-PAGE of ^{32}P-labeled trapped complexes of NEIL2 (*lane 2*) and NEIL1 (*lane 4*) along with the N terminus-tagged NEIL2 mutant (*lane 3*) with AP site-containing oligo. WT NEIL2 formed a trapped complex indicating that recombinant NEIL2 is active as an AP lyase. However, lack of such a trapped complex with the N-terminal fusion mutant of NEIL2 strongly suggests that Pro-1 is its active site.

Analysis of NEIL2 Activity with Duplex Oligo Substrates—NEIL2 was active in excising several cytosine-derived lesions with robust activity for 5-OHU and lower activity on DHU (Fig. 2*A*) when the same oligo sequence was used. Fig. 2*B* shows the ability of NEIL2 to excise 5-OHU nearly to completion in a dose-dependent fashion. NEIL2 had low activity for 5-OHC, very weak activity for thymine glycol and 8-oxoguanine, and none for U, 2-hydroxy-A, hypoxanthine, or xanthine. As expected, NEIL2 was highly active on an AP site (data not shown). A comparison of the mobility of NEIL2 incision products (*lanes 2* and *3*) with those of NTH1 (*lane 4*) and NEIL1 (*lane 5*) shows that NEIL2 carries out βδ-elimination like NEIL1, while all three are active in excising 5′-OHU (Fig. 2*A*). Thus both NEIL1 and NEIL2 are human orthologs of *E. coli* MutM/Nei because of their common reaction mechanism and conserved active site. However, the substrate preference of NEIL2 was distinct from that of NEIL1 or of the bacterial enzymes.

We compared the effect of the opposite base, G, A, or T, which are known to mispair with 5-OHU, on NEIL2 and NEIL1 activity (Fig. 2*C*). NEIL1 was more active with 5′-OHU when paired with A or T (*lanes 5* and *6*) and had weak activity with the 5-OHU·G pair (*lane 7*). In contrast, NEIL2 had significantly

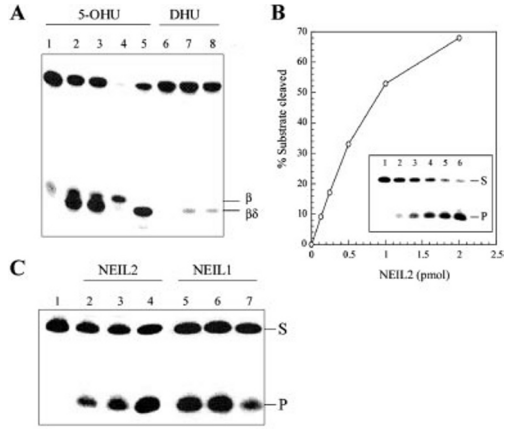

FIG. 2. *A*, incision activity of purified NEIL2. NEIL2 (0.25 pmol) was incubated with 0.1 pmol of 5′ ^{32}P-labeled duplex oligos containing 5-OHU·A (*lane 2*), 5-OHU·G (*lane 3*), DHU·A (*lane 7*), or DHU·G (*lane 8*). *Lanes 4* and *5*, incision activity of NTH1 and NEIL1 with 5-OHU·A-containing oligo. *Lanes 1* and *6*, no protein. Positions of β- and βδ-elimination products are indicated. *B*, dose-dependent activity of NEIL2 with 5-OHU·G-containing oligo. NEIL2 (*lanes 1–6*, 0, 0.125, 0.25, 0.5, 1, and 2 pmol, respectively) was incubated at 37 °C for 20 min with 2 pmol of substrate DNA as indicated, and the amount of product as a function of enzyme amount is plotted. *S*, substrate, *P*, product. *C*, complementary base-specific activity of 0.25 pmol of NEIL2 (*lanes 2–4*) and NEIL1 (*lanes 5–7*) with 2 pmol of 5-OHU-containing duplex oligo. *Lane 1*, no protein; *lanes 2* and *5*, 5-OHU·A; *lanes 3* and *6*, 5-OHU·T pair; *lanes 4* and *7*, 5-OHU·G pair.

higher activity with the 5-OHU·G pair (*lane 4*) relative to other 5-OHU pairs (*lanes 2* and *3*).

Tissue-specific and Cell Cycle-dependent Expression of Human NEIL2—Northern analysis indicated strong tissue specificity of NEIL2 expression (Fig. 3*A*). Its highest expression was found in the skeletal muscle and testis with moderate expression levels in the brain and heart. Surprisingly a very low level of NEIL2 was expressed in the other tissues tested.

The cell cycle dependence of NEIL2 expression was also examined by Northern analysis with total RNA isolated from synchronized human diploid fibroblasts. It is evident from Fig. 3*B* that the NEIL2 mRNA level did not significantly change through the cell cycle.

Nuclear Localization of NEIL2—PSORT II analysis of the sequence of NEIL2 predicted that NEIL2 could be mitochondrial rather than nuclear (34.8%, mitochondrial; 26.1%, cytoplasmic; 17.4%, nuclear; based on *k*-nearest neighbor algorithm, psort.nibb.ac.jp/). We, therefore, experimentally determined the intracellular distribution of this enzyme by using transiently expressed FLAG-tagged NEIL2 in mouse fibroblasts (Fig. 4). Contrary to the PSORT II prediction, the majority of NEIL2-FLAG protein was, however, found to be present in the nuclei of transfected cells, although a small fraction was also observed in the cytoplasm (Fig. 4*A*). In the absence of complete colocalization of mitochondria-specific MitoTracker and FLAG in the cytoplasm (Fig. 4*B*), it is evident that the ectopically expressed protein was not exclusively mitochondrial. However, it could still be present in mitochondria to carry out repair of mitochondrial DNA damage.

DISCUSSION

Characterization of DNA glycosylase/AP lyase activity in NEIL2 confirmed our prediction that both NEIL1 and NEIL2 are members of the MutM/Nei type in the mammals and utilize N-terminal Pro as the active site. However, we are surprised by the unusual specificity of NEIL2 in excising ROS-generated products of C. The rationale for the need of such a repair glycosylase may be predictable. The most frequently observed point mutation, presumably due to ROS, in aerobic organisms

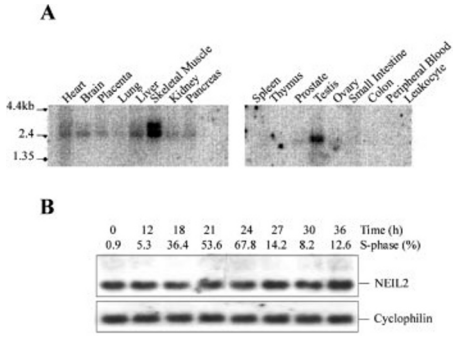

FIG. 3. *A*, tissue-specific expression of NEIL2 by Northern analysis. Blots containing 2 μg of poly(A)$^+$ RNA purified from various human tissues (CLONTECH) were probed with ^{32}P-labeled NEIL2 cDNA. *B*, cell cycle-independent expression of NEIL2. Northern analysis of *NEIL2* gene expression in synchronized MRC5 cells and the percentage of S phase cells are shown. Cyclophilin mRNA was analyzed as a control.

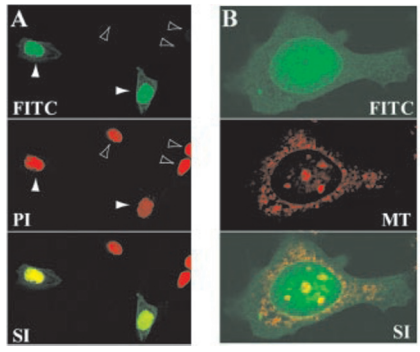

FIG. 4. **Subcellular localization of NEIL2.** *A*, the localization of NEIL2-FLAG is indicated by *FITC*, and nuclear staining is indicated by *PI*. *SI*, superimposition. Transfected (*filled arrows*) and untransfected (*open arrows*) cells are indicated. *B*, a transfected cell stained with MT superimposed with FITC fluorescence (*SI*).

is GC → AT transition (16), which could arise from mispairing of cytosine-derived lesions with adenine. Oxidation of cytosine gives rise to cytosine glycol, an unstable DNA lesion that can either be dehydrated to 5-OHC or be deaminated to uracil glycol. 5-OHU arises from either deamination of 5-OHC or dehydration of uracil glycol (17, 18). DHU, another C-derived product, is generated by ionizing radiation under anoxic condition (19). In any case, 5-OHU is quite abundant, and its level in DNA from mammalian tissues and human cells is comparable to that of 8-oxoguanine (20). 5-OHU pairs with A during DNA replication by Klenow polymerase, which will cause GC → AT transition (21). In contrast, the mammalian bypass DNA polymerase Polι preferentially incorporates T and to a lesser extent G opposite 5-OHU (19). The T·5-OHU pair would result in GC → TA transversion as frequently observed *in vivo* (22). In contrast, only the correct nucleotide G is inserted opposite 5-OHC (19). Furthermore, oxidatively modified deoxynucleotides could be incorporated into DNA from the nucleotide pool, and 5-OHdUTP is an efficient substrate for Klenow polymerase (21).

Surprisingly, while 5-OHU is the preferred substrate of NEIL2, three other human enzymes, namely NEIL1, NTH1, and uracil-DNA glycosylase, are also able to excise this lesion from DNA (10, 23, 24). This raises the possibility that 5-OHU, an important mutagenic lesion generated in significant amount in the genome, requires several back-up enzymes for its repair. Furthermore, the patterns of tissue-specific expression of NEIL1, NEIL2, and NTH1 are distinct. For example, NEIL1 expression is high in the liver, thymus, and pancreas and low in the muscle and testis (10), whereas NEIL2 mRNA level was

high in the skeletal muscle and testis (Fig. 3*A*). NTH1 expression is also high in the liver and low in the muscle (25). These results suggest that NEIL2 complements NEIL1 and other proteins in repair of 5-OHU in a tissue-specific manner. The lack of cell cycle dependence of NEIL2 mRNA, at least in human fibroblasts, shows that NEIL2 is very different from NEIL1 whose expression is significantly enhanced during the S phase (10). This suggests that NEIL2 is active on nonreplicating genomes for global repair. If the NEIL2 polypeptide turns out to parallel its mRNA level in a tissue-specific fashion (Fig. 3*A*), it will be interesting to explore the *in vivo* function of this enzyme in various tissues by examining the phenotype of the *NEIL2*-null mouse.

The physiological implications of our observation that NEIL1 was more active with the 5-OHU·A(T) pair and had weak activity with the 5-OHU·G pair, while the opposite was true for NEIL2, is not clear. It is possible that these enzymes have distinct strand specificity. NEIL2 may act on 5-OHU, derived from C in the nonreplicative genome, which is always paired with G. In contrast, NEIL1 with S phase-specific expression may be active on 5-OHU in the nascent strand when paired with A (10).

Finally, the discovery of NEIL1 and NEIL2, which generate 3′-phosphate residues at the lesion site, raises the possibility of a novel process in the BER pathway. Because human APE1, unlike its *E. coli* ortholog Xth, has extremely weak activity for removing 3′-phosphate (26), while polynucleotide kinase, present in mammalian cells but not in *E. coli*, is an efficient 3′-phosphatase (27). We have argued that NEIL1 utilizes a base excision repair subpathway, which is independent of APE1 (28). It now appears that NEIL2 shares the APE1-independent BER process with NEIL1.

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