

Human Nei-like protein NEIL3 has AP lyase activity specific for single-stranded DNA and confers oxidative stress resistance in *Escherichia coli* mutant

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Oxidative base damage leads to alteration of genomic information and is implicated as a cause of aging and carcinogenesis. To combat oxidative damage to DNA, cells contain several DNA glycosylases including OGG1, NTH1 and the Nei-like proteins, NEIL1 and NEIL2. A third Nei-like protein, NEIL3, is composed of an amino-terminal Nei-like domain and an unknown carboxy-terminal domain. In contrast to the other well-described DNA glycosylases, the DNA glycosylase activity and *in vivo* repair function of NEIL3 remains unclear. We show here that the structural modeling of the putative NEIL3 glycosylase domain (1–290) fits well to the known *Escherichia coli* Fpg crystal structure. In spite of the structural similarity, the recombinant NEIL3 and NEIL3(1–290) proteins do not cleave any of several test oligonucleotides containing a single modified base. Within the substrates, we detected AP lyase activity for single-stranded (ss) DNA but double-stranded (ds) DNA. The activity is abrogated completely in mutants with an amino-terminal deletion and at the zinc-finger motif. Surprisingly, NEIL3 partially rescues an *E. coli nth* mutant from hydrogen peroxide sensitivity. Taken together, repair of certain base damage including base loss in ssDNA may be mediated by NEIL3.

Introduction

DNA glycosylase is a key enzyme that recognizes and removes modified base in DNA and initiate base excision repair (BER) (Lindahl & Wood 1999; Nilsen & Krokan 2001; Takao & Yasui 2005). For oxidative base damage, the mammalian DNA glycosylases, OGG1 and NTH1, which remove principally oxidized purines and oxidized pyrimidines, respectively. In addition, three proteins with homology to the bacterial Nei-like glycosylases, NEIL1, NEIL2 and NEIL3, have been discovered in searches of cDNA and genome databases (Bandaru *et al.*

2002; Hazra *et al.* 2002a; Morland *et al.* 2002; Takao *et al.* 2002a). These three proteins show weak homology with each other, but contain sequences conserved with bacterial Nei and Fpg glycosylases within the catalytic and DNA-binding modules. NEIL homologues are found only in databases for vertebrate species, suggesting a role in the functions necessary in higher eukaryotes.

The substrate specificity of NEIL1 overlaps that of NTH1, and thymine glycol (Tg) and 5-hydroxyuracil (OHU) are recognized by the proteins. In contrast to NTH1, NEIL1 partly catalyzes repair of 8-oxoguanine (8-oxoG) as well (Hazra *et al.* 2002a; Morland *et al.* 2002), whereas NEIL2 acts on certain oxidized products of pyrimidines (Hazra *et al.* 2002b). As mice deficient in NTH1 do now show any apparent phenotypic abnormalities (Takao *et al.* 2002b), NEIL1 is considered to be a back-up glycosylase for NTH1 in mice (Takao *et al.* 2002a). NEIL1 and NEIL2 remove oxidative

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damage, not only from double-stranded DNA (dsDNA) but also single-stranded DNA (ssDNA) (Takao *et al.* 2002a; Dou *et al.* 2003), whereas OGG1 and NTH1 act only on dsDNA. It has been suggested that they play a role in replication-associated repair or transcription-coupled repair (Dou *et al.* 2003) although the actual role and contribution of ssDNA BER in cells is still unclear. The mode of action of OGG1 and NTH1 is bifunctional, where the apurinic/apyrimidinic (AP) site resulting from the glycosyltic removal of a damaged base is further catalyzed to produce a ssDNA nick on the damaged strand by a β -elimination reaction. This reaction leaves 3'-unsaturated sugar residue and a normal 5'-phosphate. The former residues are removed by the major human AP endonuclease, APE1. In contrast, NEIL1 and NEIL2 catalyze a further δ -elimination reaction which trims the 3'-sugar residues and leaves 3'-phosphate. The phosphate is considered to be removed by the 3'-phosphatase function of APE1, or polynucleotide kinase phosphatase. polynucleotide kinase phosphatase physically interacts with X-ray repair complementing 1 protein, which binds to polymerase β and Ligase III α , and is recognized as a molecular scaffold protein in DNA single-strand break repair (Caldecott, 2003). In the light of this interaction, the repair pathway involving NEIL1 and/or NEIL2 glycosylase has been vigorously studied (Camplans *et al.* 2005; Das *et al.* 2007; Guan *et al.* 2007; Dou *et al.* 2008).

The third Nei-like protein, NEIL3, is much less characterized than NEIL1 and NEIL2. NEIL3 has N-terminal Nei-like domain and an extra C-terminal domain. We have examined the DNA glycosylase activity of reticulocyte-translated NEIL3 toward Tg- and 8-oxoG-containing substrates and failed to detect any activity (Takao *et al.* 2002a). Another group has demonstrated that an extract from insect cells expressing human NEIL3 in a baculovirus expression system showed an excision activity for formamidopyrimidine (Fapy) residues, apparently higher than found in uninfected insect extract, but failed to detect the activity *in vitro* using recombinant protein from *E. coli* (Morland *et al.* 2002). Although they suggested that *in vivo* modification might be required for the activity of NEIL3, the DNA glycosylase activity and its repair function *in vivo* remain to be confirmed.

In this paper, we have characterized recombinant NEIL3 protein purified from *E. coli*, demonstrating its DNA-binding activity and AP-site nicking activity. A significant feature is that these activities are highly specific for ssDNA. We have further found that oxidative stress-sensitive *E. coli nth nei* could be partially rescued by NEIL3, suggesting *E. coli*-produced NEIL3 has a repair

function for certain oxidative DNA lesions and/or base loss in ssDNA.

Results

Structural similarity between the NEIL3 glycosylase domain (NEIL3GD) and Fpg

NEIL3 consists of a putative N-terminal glycosylase domain (1–290; NEIL3GD) and a unique C-terminal domain (291–607; NEIL3CTD) (Fig. 1A). The latter domain shows no overall homology to known proteins, whereas it contains a zinc-finger motif and a short sequence homologous to an uncharacterized part of TOPOIII and APEXL2 (Tsuchimoto *et al.* 2001). NEIL3GD has helix-two-turn-helix (H2TH) and zinc finger (ZnF) motifs that are the hallmarks of Fpg-Nei glycosylase family (Sugahara *et al.* 2000) such as *E. coli* Fpg. The sequence identity to *E. coli* Fpg in the remaining part is, however, relatively weak, and the amino terminal proline which is strictly conserved in bacterial Fpg, Nei and vertebrate NEIL1 and NEIL2 glycosylases is replaced by valine in NEIL3. As the N-terminal proline is known to serve as the catalytic residue for DNA glycosylase function, it makes the enzymatic function of NEIL3 obscure. Thus we first tested the validity of NEIL3 as a DNA glycosylase enzyme by building a structural model. In addition to mammalian species, several vertebrate sequences for NEIL3 were found in EST and genome databases, including mammals, chicken, frog, and fish. We found that a 21-amino-acid stretch near the N-terminus of mammalian NEIL3 is absent in the frog (*Silurana tropicalis*) and fish (*Fugu rubripes*) counterparts, as illustrated by the alignment shown in Fig. 1B (human versus *S. tropicalis* sequence). The secondary structure prediction for human NEIL3 without the 21 residues (Q36 to L56) becomes very similar to that of *S. tropicalis* NEIL3 and the known structure folding of *E. coli* Fpg (Fig. 1B). Using the *E. coli* Fpg structure data as a template, an energy minimized model for human NEIL3GD was obtained (Fig. 1C). According to the model structure, positions of possible DNA interacting segments (i.e., turns between β 2– β 3, β 4– β 5, β 7– β 8, H2TH motif and ZnF motif) are predicted. Therefore, NEIL3GD may at least be a DNA interacting protein even though the N-terminal catalytic residue is altered.

Purification of recombinant proteins and single-stranded DNA-binding ability of NEIL3GD

We have found that NEIL3 produced by an *in vitro* translation system shows no glycosylase activity toward

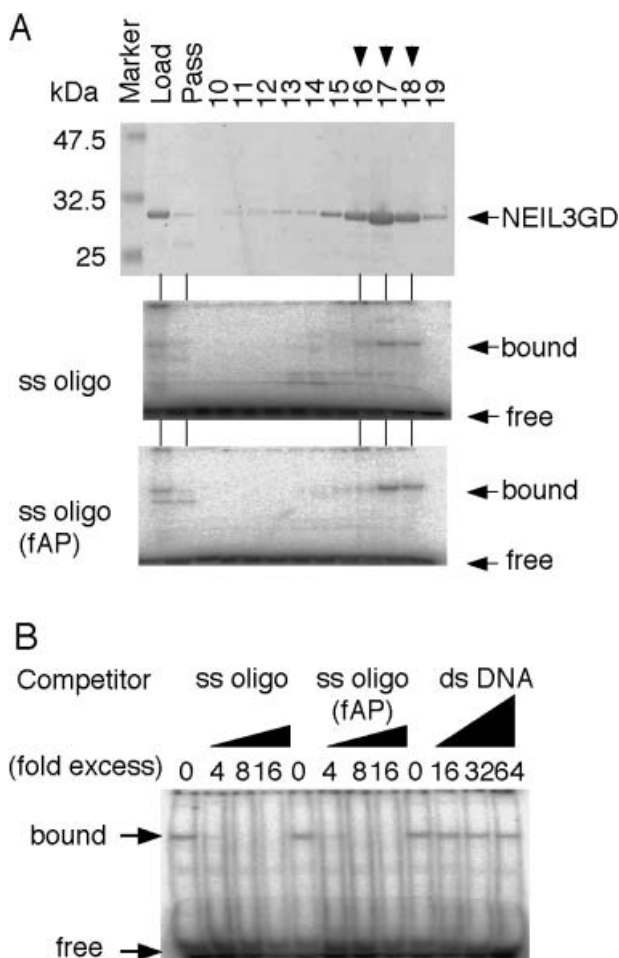


Figure 2 DNA-binding ability of purified NEIL3GD. (A) Heparin column purification of NEIL3GD and its DNA-binding ability to ssDNA. NEIL3GD was eluted mainly in fraction numbers 16, 17 and 18 as shown in Coomassie Brilliant Blue stained gel (upper panel). The binding with 5'-labeled undamaged single-stranded 30 mer oligonucleotide probe was analyzed by EMSA (middle panel). Single-strand oligonucleotide containing an AP site analog, tetrahydrofuran (fAP), was similarly analyzed (lower panel). The probe was incubated with protein on ice for 30 min and run on a native acrylamide gel. (B) Competition EMSA. The NEIL3GD was pre-incubated with the undamaged ssDNA probe and unlabeled competitors (ss oligo, ss oligo (fAP) and dsDNA, as shown) and mixed with labeled probe as shown.

double-stranded Tg and 8-oxoG substrates (Takao *et al.* 2002a). However, the structural similarity suggests that NEIL3 may have other enzymatic features of Fpg-Nei glycosylase proteins such as DNA-binding or AP-lyase activities. To obtain more biochemical information, we generated full-length NEIL3 and NEIL3GD by expressing C-terminally 6xHis-tagged proteins in *E. coli*. Although NEIL3GD could be purified to near homogeneity, there

were some difficulties regarding the expression and purification of the full-length NEIL3 protein. The yield and purity of soluble NEIL3 were less than NEIL3GD. The affinity-purified NEIL3 fraction contained polypeptides corresponding to the full-length protein (68 kDa) and its degradation product (50 kDa) as well as several bacterial protein contaminants as judged by SDS-PAGE and western blot analysis with α -6xHis antibody (a purity of the full-length NEIL3 polypeptide is approximately 20%). Thus, for the basic characterization we examined whether NEIL3GD possessed the characteristics of the full-length protein, and in some experiments described below, we show data obtained from highly purified NEIL3GD.

The affinity-purified NEIL3GD fraction was subsequently subjected to heparin column chromatography. NEIL3GD eluted at 250 mM NaCl was readily visualized by Coomassie Brilliant Blue staining of SDS-PAGE (Fig. 2A, upper panel). However, these fractions did not bind to dsDNA (not shown). Instead, NEIL3GD showed binding activity to ssDNA (Fig. 2A, middle panel) as well as ssDNA containing an AP site analog, tetrahydrofuran (fAP), which is resistant to AP lyase-mediated strand incision (24) (Fig. 2A, lower panel). The specific binding of NEIL3GD to ssDNA was further confirmed by a competition assay (Fig. 2B). In this experiment, a 64 times molar excess of dsDNA did not compete with the single-stranded probe, indicating that the DNA binding of NEIL3GD is highly specific for the single-stranded form.

NEIL3 has AP lyase activity on ssDNA

Glycosyltic release of oxidized bases by the Fpg-Nei glycosylase family is followed by the β - δ elimination reaction of the associated AP lyase, resulting in a nick on the damaged strand. In the expectation that NEIL3 may have AP lyase activity, we carried out a DNA nicking assay with 30-mer oligonucleotide (oligo) containing a single AP site. When the single-stranded AP substrate (ssAP) was incubated with NEIL3GD or full-length NEIL3, products nicked at the AP site were observed with time (Fig. 3A or B, respectively). A double-stranded AP substrate (dsAP) was not susceptible to the AP lyase activity of NEIL3 (not shown). In a high-resolution sequencing gel, the products nicked by NEIL3GD were those of both β - and δ -elimination (Fig. 3C). Therefore, NEIL3 has an incomplete but a potential activity of the β - δ elimination reaction. NEIL3 showed lower nicking activity at the AP site than NEIL1 that was demonstrated with the identical substrate (Takao *et al.* 2002a). To avoid misinterpreting contaminating bacterial activities as

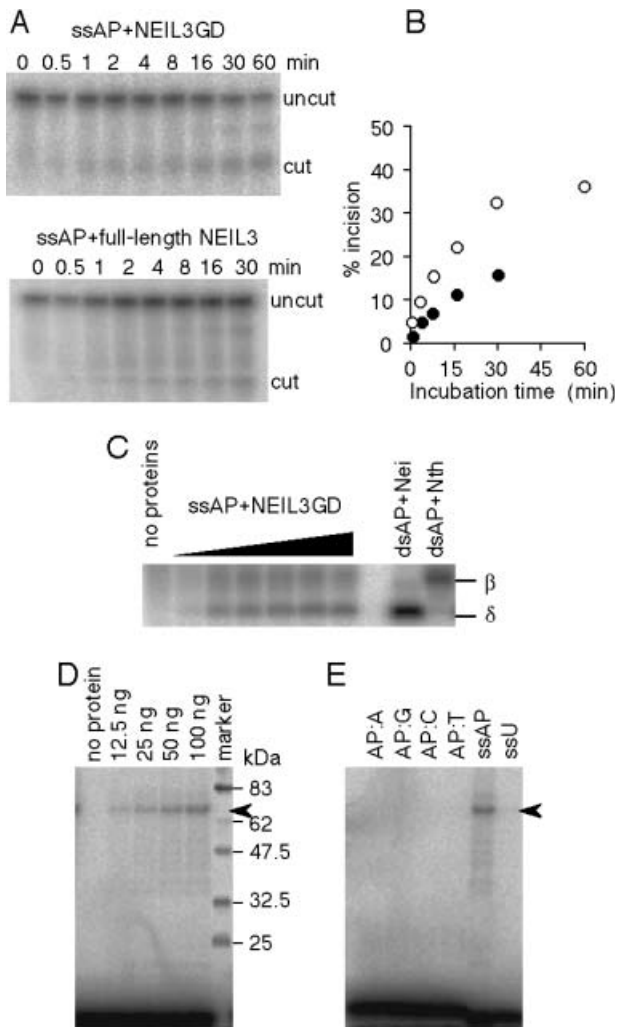


Figure 3 ssDNA specific AP lyase activity of NEIL3. (A) NEIL3 incises ssAP site. In total 100 ng of NEIL3GD (upper) or 400 ng of full-length NEIL3 (lower) was incubated with a single-stranded, natural AP site-containing 30 mer oligonucleotide. The incision products were separated on denaturing polyacrylamide gel. (B) Plot of incision products vs. incubation time. Open and closed circles represent densitometric data of NEIL3GD and full-length NEIL3 obtained from A, respectively. (C) High resolution sequence gel analysis of incision product. NEIL3GD of 0–200 ng was reacted with ssAP probe. The control reaction with Nei or Nth is shown for β - δ elimination or β elimination, respectively. (D) SDS-PAGE analysis of DNA-NEIL3 cross-link. Full-length NEIL3 was mixed with a ssAP-containing oligonucleotide probe in the presence of sodium borohydride to trap the AP lyase reaction intermediate as mentioned in Experimental procedures. (E) NEIL3 specifically forms cross-linked product to ssAP. The trapping assay was carried out as in D. dsAP substrates were prepared by annealing with the complementary strand having the base shown opposite the AP site. ssU denotes the uracil-containing single-stranded oligonucleotide, a precursor of ssAP, being treated by UDG.

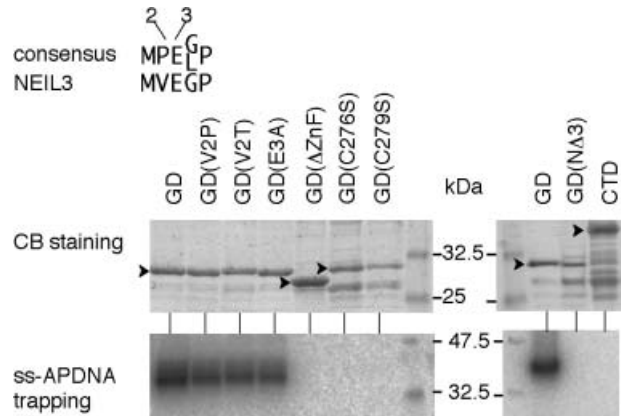


Figure 4 Determinants for NEIL3 AP lyase activity. A consensus N-terminal sequence of known Fpg, Nei glycosylases is compared with the NEIL3 counterpart (upper panel). Proteins having a point mutation or deletion, with a C-terminal 6xHis tag, were purified as shown in a Coomassie Brilliant Blue stained SDS-gel. The protein samples were analyzed by the borohydride-trapping assay.

the NEIL3 activity, we conducted a borohydride-trapping assay that detects a cross-linked product between a labeled DNA and protein, and can identify the protein species cross-linked to the probe on SDS-PAGE. The labeled ssAP substrate was incubated with the fraction containing full-length NEIL3 in the presence of NaBH_4 . As shown in Fig. 3D, the DNA-NEIL3 complex alone was detected at the expected size position, whereas any complex with bacterial enzymes, if contaminated, should appear at lower than 50 kDa. Interestingly, the cross-link did not occur with double-stranded AP (dsAP) substrate paired with any nucleotide opposite the AP site (Fig. 3E). The ssDNA-specific AP lyase activity coincides with the ssDNA-specific DNA-binding activity (Fig. 2).

Requirement of amino-terminal residues and ZnF motif for AP lyase

An enigmatic feature of NEIL3 is its amino terminal valine residue, while proline is strictly conserved among members of the Fpg-Nei glycosylase family and is essential for their glycosylase function. The third glutamic acid is also strictly conserved in the Fpg-Nei glycosylase family. It is, however, reported that substitution of the N-terminal Pro2 or Glu3 in bacterial Fpg or Nei does not destroy the AP lyase activity although the glycosyltic activity is lost (Lavrukhin & Lloyd 2000; Sidorkina & Laval 2000; Burgess *et al.* 2002). We evaluated the requirement of NEIL3 N-terminal residues for the AP lyase function (Fig. 4). In the borohydride-trapping assay, alteration of

Table 1 Test oligonucleotides for NEIL3

	Ss oligo	Ds oligo (paired with)
Tg (5S)	–	– (A)
Tg (5R)	–	– (A)
OHU	–	– (A,G)
DHT	–	– (A)
HMU	–	– (A)
8-oxoG	–	– (C, A)
2-oxoA	–	– (G, T)
adenine	ND	– (8-oxoG)
O ⁶ meG	–	– (C)
εA	–	– (T)
Uracil	–	– (A, G)
Mismatch	–	– (any combination)
AP	+	– (any combination)

Abbreviations: Tg (5S), thymine glycol (5S,6R); Tg (5R), thymine glycol (5R,6S); OHU, 5-hydroxyuracil; DHT, 5,6-dihydrothymine; HMU, 5-hydroxymethyluracil; 8-oxoG, 7,8-dihydro-8-oxoguanine; 2-oxoA, 1,2-dihydro-2-oxoadenine; O⁶meG, 6-O-methylguanin; εA, 1,-N6-ethenoadenine.

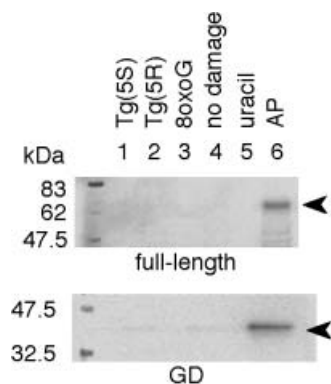


Figure 5 Example of oxidative lesions insensitive to NEIL3. Borohydride-trapping assay with full-length NEIL3 (upper panel) or NEIL3GD (lower panel) is shown. The NEIL3 and NEILGD formed cross-links only with the AP-site-containing oligo (lane 6).

Val2 into the consensus proline context, NEIL3GD(V2P), and another alteration, NEIL3GD(V2T), retained the trapping activity. Similarly, a point mutant at Glu3, NEIL3GD(E3A), showed a trapped product. The residual activities in these mutants seem to imply less relevance of Val2 and Glu3 to the AP lyase activity of NEIL3 as is the case of Fpg and Nei mutants. In contrast to these amino acid substitutions, truncation of Val2 and Glu3, NEIL3GD(NΔ3), completely abolished the activity, suggesting that the appropriate length of the N-terminus seems to be required to keep the structure of the active site pocket. In addition to creating the N-terminal

modifications, we disrupted the C-terminal ZnF motif. This motif in Fpg is an essential part of the DNA-binding region including Arg259 (Arg271 in NEIL3), which interacts directly with the damaged site. A deletion of the ZnF motif, NEIL3GD(ΔZnF), or substitutions of cysteine in the motif into serine, NEIL3GD(C276S) or NEIL3GD(C279S), resulted in complete loss of AP lyase activity. Mutation analysis mentioned above excludes a possibility that the recombinant polypeptide might invoke a non-catalytic reaction at the AP site (some basic molecules make cleavage at the site). The data rather imply that AP lyase activity of NEIL3GD is resulted from a correct protein folding, and support the validity of our structural modeling (Fig. 1C).

Substrate specificity

Several oxidative and some other base modifications at a defined position in the chemically synthesized 30 mer oligo were subjected to the trapping assay with the recombinant full-length NEIL3 and NEIL3GD. However, we were unable to find a substrate for NEIL3 among them in either the single-stranded or double-stranded form except for ssAP (Table 1 and Fig. 5). For example, Tg (either 5S or 5R isomer), a substrate extensively examined for Nth, Nei and NEIL1 (Katafuchi *et al.* 2004; Miller *et al.* 2004), was not catalyzed as demonstrated in Fig. 5 (lanes 1 and 2). Neither was a representative oxidative purine lesion, 8-oxoG, which is a good substrate for Fpg and OGG1 (lane 3).

Partial complementation of *Escherichia coli* hydrogen peroxide sensitivity by NEIL3

Although we were not able to identify a substrate for DNA glycosylase activity *in vitro*, NEIL3 may play a certain repair role *in vivo*. To evaluate the *in vivo* function of NEIL3, we introduced NEIL3 cDNA into a *E. coli nth nei* double mutant strain which is sensitive to hydrogen peroxide (Saito *et al.* 1997). The vector used has a *lac* operon with a *tac* promoter. We applied a non-induced condition for the transformed cell culture to allow cells expressing mildly in the strain as mentioned (Aburatani *et al.* 1997). As shown in Fig. 6, *E. coli* with the vector alone showed no increase in resistance to hydrogen peroxide, while the full-length NEIL3 partially rescued the sensitive phenotype of the strain. The result was highly reproducible. Expression of NEIL3CTD did not change the sensitivity indicating that the CTD did not confer the resistant phenotype (not shown). These results strongly suggest that NEIL3 repairs a subset of oxidative base damage induced by hydrogen peroxide in *E. coli*.

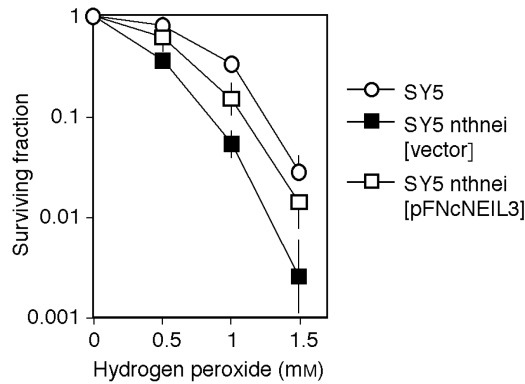
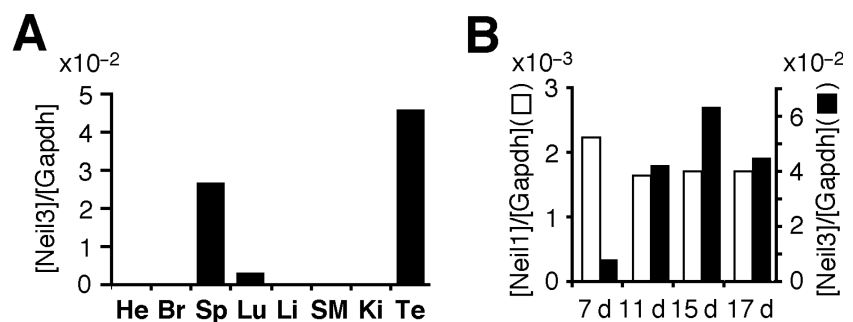


Figure 6 Partial complementation of hydrogen peroxide sensitivity by NEIL3. The SY5 *nth nei* mutant strain containing vector alone (open squares) shows greater sensitivity to hydrogen peroxide than the parental wild-type strain SY5 (open circles). The transformant with *NEIL3* (pFncNEIL3, closed squares) partially rescues the sensitive phenotype of SY5 *nth nei*.

Tissue and embryonic expression of mouse Neil3

We have previously examined mouse Neil1 expression in various tissues (Takao *et al.* 2002a). Using the same cDNA panel derived from BALB/c males (ages 8–12 weeks), tissue specific expression of mouse Neil3 was examined as shown in Fig. 7A (i.e., comparable with the previous data in Takao *et al.* 2002a). The expression was high in testis and spleen, and undetectable in many tissues, indicating a selective expression of mNeil3. In accordance with the results, Northern blot analysis of human NEIL3 has revealed the selective expression in testis and thymus (Morland *et al.* 2002). Embryonic expression of mNeil1 and mNeil3 was also examined (Fig. 7B). Although mNeil1 is constitutively expressed, mNeil3 shows increase in the expression level up to 15d and decline thereafter. The results suggest that the expression of mNeil3 depends on the developmental stage.

Figure 7 Tissue and embryonic expression. (A) Selective tissue expression of mouse Neil3. An equalized cDNA panel (Clontech) was used for real-time, Q-PCR. He, heart; Br, brain; Sp, spleen; Lu, lung; Li, liver; SM, skeletal muscle; Ki, kidney; Te, testis. (B) Stage-dependent expression of mNeil3 in embryos. A mouse embryonic cDNA panel (Clontech) was used for the evaluation of mNeil1 and mNeil3 expressions. The data were normalized by [*Gapdh*].



Discussion

In this study we have characterized NEIL3 as an AP lyase *in vitro* and a potent DNA repair protein *in vivo*. The unique characteristic of NEIL3 as compared with other Fpg, Nei and mammalian NEIL proteins is its ssDNA-specific binding and enzymatic activity. In a structural model, we have demonstrated that NEIL3GD shows overall similarities to *E. coli* Fpg. According to the crystal structure of dsDNA–*E. coli* Fpg complex (Gilboa *et al.* 2002), the amino acid residues interacting with the damaged DNA strand are predicted to be Lys57, His71, Asn169, Arg259. These amino acids are conserved in the 3D structural model of human NEIL3GD. In contrast, the amino acids in the *E. coli* Fpg protein (Ser105, Val89, Arg91) which are assigned to the interaction with the opposite strand are replaced by other amino acids in NEIL3. It is reported that mammalian NEIL1 and NEIL2 whose amino acids corresponding to the opposite strand-interacting residues of Fpg are also not conserved, can remove damage from ssDNA in addition to dsDNA. (Takao *et al.* 2002a; Dou *et al.* 2003). Thus, the NEIL family may less require dsDNA *per se*, and NEIL3 may have evolved as a DNA-interacting protein exclusive of a double-stranded form.

NEIL3 has a long C-terminal sequence. Other members of the Fpg–Nei glycosylase family do not show such a C-terminal domain. Because the C-terminus of the 3D structural model of NEIL3GD is extruding from the core structure, NEIL3CTD is expected to form an additional domain structure. It would be of interest to know if NEIL3CTD might regulate the enzyme activity or function to direct the appropriate repair *in vivo* via interacting with other proteins.

Morland *et al.* (2002) showed that NEIL3 expressed by baculovirus system excised Fapy residues while NEIL3 expressed by *E. coli* system did not. In preliminary experiments, we failed to detect the incision activity of NEIL3 to methyl-Fapy, a methylated analog of Fapy, synthesized according to Asagoshi *et al.* (2000). Although

one candidate for the NEIL3 substrate may be natural Fapy residues, *in vivo* complementation using *E. coli ntl1* suggests that NEIL3 can repair minor but lethal damage rather than Fapy because the strain retains intact Fpg and UvrABC which removes Fapy residues. Further studies, identifying the substrate specificities, will be needed.

Torisu *et al.* (2005) have established *Neil3* knockout mice which are viable and fertile, and look healthy for at least 24 weeks after birth. Northern blot analyses of human and mouse have shown by them and others (Morland *et al.* 2002), indicating a specific expression in hematopoietic tissues. We also examined the tissue specificity for mouse *Neil3* expression by quantitative (Q)-PCR and obtained similar results except for an abundant expression in testis where Torisu *et al.* (2005) barely detected in testis. As their mRNA and our cDNA were supplied by the same company (Clontech), the difference might be lot-to-lot variation. In any case, high expression of human *NEIL3* in testis was reported by them and Morland *et al.* (2002). More recently, the details for several DNA glycosylases in brain by an *in situ* hybridization analysis have been demonstrated (Rølseth *et al.* 2008). The RNA for *mNeil3* was detected in stem cell enriched regions of young mice and decreased with age (undetectable in brain 4 weeks after birth by Northern blot analysis). We further showed that the *mNeil3* expression is dependent on the embryonic stage in contrast to the constitutive expression of *mNeil1*. The tissue-specific and development- and age-dependent expression patterns of NEIL3 are a unique character and might suggest an additional physiological role in such NEIL3-expressing cells and tissues.

Experimental procedures

Synthetic oligonucleotide substrates

The phosphoramidite chemicals for modified bases were obtained from Glen Research and the oligos containing a modified base were synthesized and purified. Those include fAP, OHU, 5,6-dihydrothymine (DHT), 5-hydroxymethyluracil (HMU), 8-oxoG, 2-oxoadenine (2-oxoA), O⁶-methylguanine (O⁶meG), ethenoadenine (εA), or uracil (U). Oligo containing Tg stereoisomers, 5S-6R (Tg(5S)) or 5R-6S (Tg(5R)) are synthesized as described (Iwai 2001). The sequence of the substrate is 5'-CTCGTCAGCATCTXCATCATAACAGTCAGTG, where X is a modified base. The oligo was labeled using T4 polynucleotide kinase (Takara) with [γ -³²P] ATP (5000 Ci/mmol; Amersham Biosciences). A substrate containing a natural AP site was made by incubating the uracil-containing oligo with uracil DNA glycosylase, UDG (Invitrogen).

Protein modeling

The amino acid sequences of *S. Tropicalis* NEIL3 and human NEIL3 were sent to 3D-pssm website (www.sbg.bio.ic.ac.uk/servers/3dpssm/). Based on the predicted secondary structures, a sequence alignment between NEIL3 proteins and *E. coli* Fpg was made. The structure of *E. coli* Fpg (MutM, Protein Data code: entry 1k82 chain A) (Gilboa *et al.* 2002) was then used as a template for the homology modeling. The result of CLUSTAL Multiple Sequence Alignment was checked and repeated with the human NEIL3GD deletion model (21 amino acids, Q36 to L56). The substituted model coordinates were submitted to the 3D-pssm. The substitution from Fpg to the NEIL3 was carried out with program O (Jones *et al.* 1991). Deletions with respect to Fpg sequence were made interactively and energy-minimized locally using the graphic program O. The generated model was then energy minimized using the CNS software package (Brünger *et al.* 1998). The stereochemical quality of the final model was ensured using the program PROCHECK (Laskowski *et al.* 1993).

Expression and purification of recombinant NEIL3

Full-length NEIL3 and NEIL3GD (M1 to K281) cDNAs were subcloned in a pET21 vector (Novagen) to produce C-terminal 6xHis-tagged recombinant proteins. Deletion and substitution mutants were made by PCR with appropriate primers and subcloned in the same vector. All mutant clones were sequenced to verify the modifications. Production in *E. coli* BL21 CodonPlus (Stratagene) and affinity column (Ni-NTA, Qiagen) purification for the tagged protein from the lysate under native conditions was described previously (Takao *et al.* 2002a). The recombinant NEIL3GD was further purified on a HiTrap heparin HP column (Amersham) in a HPLC system (BioRad) and characterized for its DNA-binding activity and enzymatic activity.

DNA-binding assay

An electrophoretic mobility shift assay (EMSA) was used to examine the DNA-binding affinity of NEIL3. The labeled oligo was left single-stranded or annealed to the complementary strand. The heparin column fraction was incubated with the probe (5 nM) in a buffer of 25 mM HEPES-KCl (pH 7.4), 200 mM NaCl, 1 mM MgCl₂, 0.5 mM dithiothreitol, 0.01% NP-40, 4% glycerol for 30 min on ice. The protein DNA complex was separated on a 6% non-denaturing polyacrylamide gel. In competition assays, single-stranded unmodified oligo, single-stranded fAP-oligo or double-stranded DNA fragment generated by cutting plasmid DNA with *Pvu*II (i.e., blunt-ended) was preincubated on ice for 15 min as a competitor and mixed with the labeled probe as indicated above.

Incision assay and borohydride-trapping assay for AP lyase

The incision assay was carried out with the natural AP-site-containing substrate. The labeled substrate (5 fmol) was incubated

with NEIL3 (100 ng) in 5 μ L of buffer A (50 mM sodium phosphate (pH 7.5), 5 mM EDTA, 0.5 mM dithiothreitol) containing 100 mM NaCl at 30 °C for 30 min unless otherwise stated. The incision product was separated on a 20% polyacrylamide gel containing 7 M urea as described previously (Takao *et al.* 2002a). A borohydride-trapping assay (Zhang *et al.* 2000) was used to cross-link AP lyase and the labeled DNA substrate. The substrate (25 fmol) was incubated with NEIL3 (100 ng) in 20 μ L of buffer A containing 100 mM sodium borohydride at 30 °C for 30 min. Glucose (6.7%) was added to quench the reaction. A portion was mixed with SDS loading buffer and run on a 5–20% gradient SDS polyacrylamide gel. In some experiments, 8 units of Fpg (NEB) was substituted for NEIL3, as a control for the borohydride-trapping reaction.

Complementation test of *Escherichia coli* mutant

NEIL3 cDNA was subcloned into a pUC-derived bacterial expression vector pFNC (Aburatani *et al.* 1997). *Escherichia coli* SY5 *nth nei* was transformed with the construct or with empty vector. Overnight cultures of the transformants were appropriately diluted and plated (0.1 mL) on LB agar plus ampicillin, containing hydrogen peroxide (0–1.5 mM). After incubation at 37 °C for about 40 h, the number of colonies was counted to estimate survival rate. The mean values from three independent experiments were plotted.

Quantitative PCR

Q-PCR was carried out with LightCycler instrument (Roche) as described (Takao *et al.* 2002a). A mouse multiple tissue cDNA panel (200 BALB/c males, age 8–12 weeks) and embryo cDNA panel (200 Swiss Webster/NIH embryos) were purchased from Clontech and further quantitated with amplimers for glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as described previously (Takao *et al.* 2002a). Amplimers for *Neil3* are 5'-ACTGAATGGAGAGAAGATCCGGG and 5'-CAGCTCCTCCCTAA GGTTCCTCA.

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