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Deoxyribophosphate lyase activity of mammalian endonuclease VIII-like proteins

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Abstract Base excision repair (BER) protects cells from nucleobase DNA damage. In eukaryotic BER, DNA glycosylases generate abasic sites, which are then converted to deoxyribo-5′-phosphate (dRP) and excised by a dRP lyase (dRPase) activity of DNA polymerase β (Polβ). Here, we demonstrate that NEIL1 and NEIL2, mammalian homologs of bacterial endonuclease VIII, excise dRP by β-elimination with the efficiency similar to Polβ. DNA duplexes imitating BER intermediates after insertion of a single nucleotide were better substrates. NEIL1 and NEIL2 supplied dRPase activity in BER reconstituted with dRPase-null Polβ. Our results suggest a role for NEILs as backup dRPases in mammalian cells.

Keywords: DNA repair; Base excision; Deoxyribophosphate lyase; NEIL; DNA polymerase β

1. Introduction

Base excision repair (BER) is responsible for cleansing DNA of non-bulky, frequently occurring base lesions [1]. During BER, the lesion is first located by one of DNA glycosylases, enzymes that excise damaged bases. This results in an abasic (AP) site, which is hydrolyzed at its 5′-side by an AP endonuclease, leaving a nick flanked by a 3′-hydroxyl of an undamaged deoxynucleotide and a deoxyribo-5′-phosphate (dRP) to which the damaged base was formerly connected. DNA polymerase then inserts a normal deoxynucleotide; however, ligation to restore intact DNA is impossible because of the dangling dRP moiety. The situation is resolved by a special enzymatic activity, deoxyribophosphatase (dRPase), excising dRP (short-patch BER, Fig. 1), or by continuing DNA synthesis with strand displacement, followed by degradation of the displaced strand (long-patch BER). The whole process currently draws much attention due to its antimutagenic and tumor suppression role [2].

The dRPase activity, a rate-limiting step in BER [3], plays a central role in switching between its short- and long-patch branches. In *Escherichia coli*, two main dRPase activities have been observed. A Mg^{2+}-dependent RecJ deoxyribophosphodiesterase hydrolyzes the 3′-phosphodiester bond in dRP releasing deoxyribo-5′-phosphate [4]. Formamidopyrimidine-DNA glycosylase (Fpg) does not depend on Mg^{2+} and catalyzes β-elimination of dRP rather than its hydrolysis (deoxyribophosphate lyase), the product being 2-hydroxy-5-oxopent-3-enyl phosphate [5]. Both RecJ and Fpg leave a 5′-terminal phosphate in DNA, creating a substrate for DNA ligase. Little Mg^{2+}-independent activity was observed in *fpg* null cells [5], although dRPase activity *in vitro* has also been reported for *E. coli* endonuclease VIII (Nei), a homolog of Fpg [6]. Nei dRPase activity may explain the lack of phenotype in *fpg recJ* double mutants [4].

The major dRPase in mammalian cells is DNA polymerase β (Polβ) [7,8], which has a dRP lyase domain [9,10]. Polβ-deficient cells show low dRPase activity [7], but some residual dRP removal by extracts from these cells is still present [11]. A dRPase activity in vertebrates was also shown for the mitochondrial DNA polymerase γ [12–14], translesion DNA polymerases ι [15] and λ [16], and a Mg^{2+}-dependent activity was purified from human cells and calf thymus [17]. It is possible that while Polβ carries out the bulk of dRP removal, other activities could be more specifically employed for some lesions, cell or tissue types, or at certain cell cycle points.

Recently, three mammalian homologs of Fpg and Nei have been identified and termed NEIL (Nei-like, or endonuclease VIII-like)-1, -2, and -3 [18–23]. Based on the similarity of their active sites to those of Fpg and Nei (Fig. 2), one could expect that they also display dRPase activity. In this report we show that two of these proteins, NEIL1 and NEIL2, are capable of removing dRP from DNA with the efficiency comparable to that of Polβ, and that they can substitute for Polβ dRPase activity in a reconstituted BER system.

2. Materials and methods

2.1. Oligonucleotides and enzymes

ODNs were synthesized from phosphoramidite precursors (Glen Research) using established protocols. The modified 23-mer strand, 5′-CTCTCCCTTCXCTCCTTTCCTCT-3′, where X is uracil (U) or 8-oxoguanine (8-oxoG), was 5′-labeled using γ[^{32}P]-ATP and polynucleotide kinase, purified by PAGE, precipitated and annealed to a complementary 23-mer strand 5′-AGAGGAAAGGAGNGAAGGGAGAG-3′ (N = A, C, G, or T). To label the modified ODNs at the 3′-terminus, they were annealed to a 25-mer complementary strand, 5′-GTAGAGGAAAGGAGNGAAGGGAGAG-3′, and the overhang

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Abbreviations: AP, apurinic/apyrimidinic; BER, base excision repair; dRP, deoxyribo-5′-phosphate; dRPase, deoxyribo-5′-phosphate lyase; ODN, oligodeoxynucleotide; PAGE, polyacrylamide gel electrophoresis; Polβ, DNA polymerase β; mPolβ, Polβ K35A/K68A/K72A mutant; SDS, sodium dodecyl sulfate

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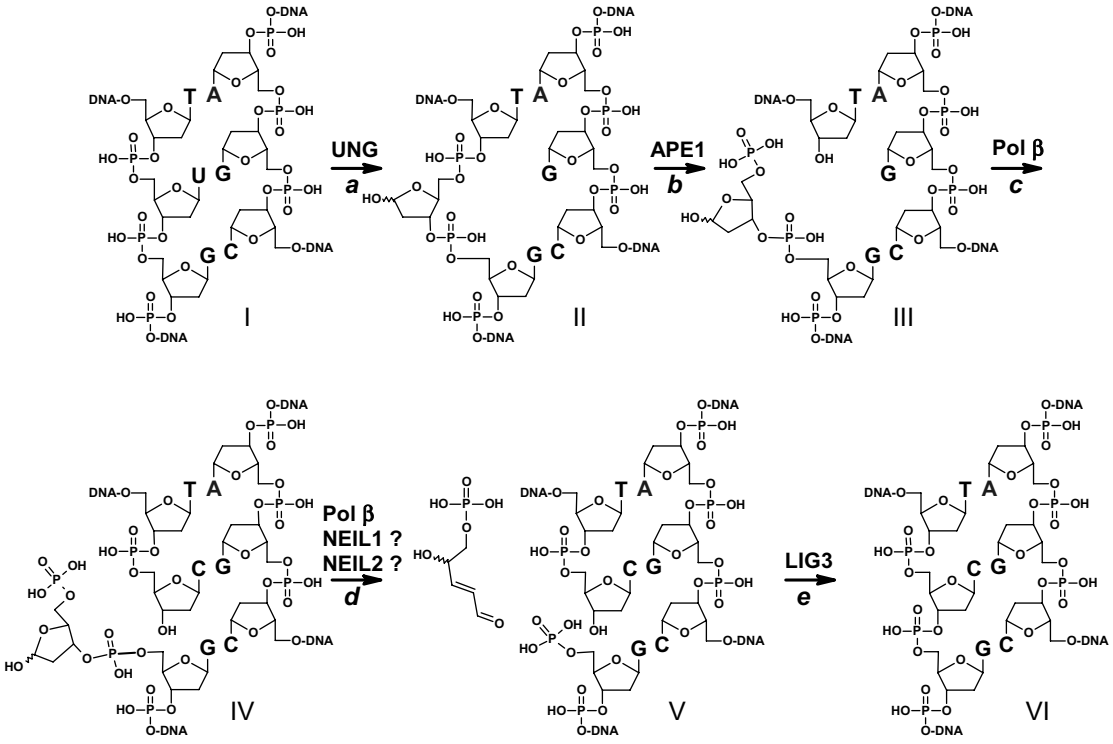

Fig. 1. General scheme of base excision repair. Main stages of the short-patch BER sub-pathway and the relevant enzymes are shown schematically for a U:G mispair (I) formed by spontaneous cytosine deamination: (a), excision of the damaged base by a DNA glycosylase (UNG) with formation of an AP site (II); (b), 5′-incision of the AP site by AP endonuclease (APE1) with formation of a dRP site (III); (c), insertion of a correct nucleotide by a DNA polymerase (Polβ for mammalian short-patch BER) with formation of a ''hanging'' dRP site (IV); (d), elimination of the dRP site by a dRP lyase (Polβ, or, possibly, NEIL1/NEIL2) with formation of a nick in DNA (V); (e), ligation of the nick by a DNA ligase (DNA ligase III for mammalian short-patch BER) and restoration of undamaged DNA (VI).

was partially filled by Klenow fragment using α[^{32}P]-dATP. T4 polynucleotide kinase, T4 DNA ligase and *E. coli* uracil-DNA glycosylase (Eco-Ung) were purchased from New England Biolabs. Exonuclease-deficient Klenow fragment was a gift from Dr. Holly Miller (SUNY Stony Brook); human uracil-DNA glycosylase (UNG) was kindly provided by Dr. Alexander Ischenko (Institut Gustave Roussy, France). NEIL1 and NEIL2 proteins, wild-type and dRPase-deficient Polβ were expressed in *E. coli* and purified as described [24–26]. 8-Oxoguanine-DNA glycosylase (OGG1) and AP endonuclease (APE1) were expressed as His_6-tagged proteins and purified using Ni^{2+}-chelate chromatography [27]. Concentrations of active forms of NEIL1 and NEIL2 were determined by $NaBH_4$-stabilized crosslinking of the enzyme (10 nM) to saturating amounts (5 μM) of a dRP substrate as described in the following section.

2.2. dRPase and crosslinking assays

To prepare a dRP substrate, the 3′-labeled U-containing duplex (20 nM unless indicated otherwise) was treated with 1 U Eco-Ung and 1 μM APE1 in 25 mM K–phosphate (pH 7.4), 5 mM $MgCl_2$ and 1 mM dithiothreitol for 10 min at 25 °C. To obtain a substrate with an inserted nucleotide, this reaction mixture was supplemented with 40 nM mPolβ and 1 mM dGTP. To analyze dRPase activity, NEIL1, NEIL2, or Polβ was added (20 μl final reaction volume) and incubated for 10 min at 25 °C. The reaction products were stabilized by 50 mM $NaBH_4$ for 30 min on ice. The reaction products were resolved by 20% denaturing PAGE and quantified using Molecular Imager FX (Bio-Rad). To analyze crosslinking, 50 mM $NaBH_4$ was added together with dRP lyases and incubated for 30 min on ice. The products were resolved by 12% SDS–PAGE and imaged as above.

2.3. Base excision repair reconstitution assay

The reaction mixtures (20 μl) included 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25 μg/ml bovine serum albumin, 10 nM substrate duplex, 1 mM dGTP, 600 nM APE1, 500 nM Polβ or mPolβ, T4 DNA ligase (1 Weiss unit), and, if needed, 75 nM NEIL1 or 150 nM NEIL2. When the repair of an AP site was reconstituted, the U-containing substrate was pre-treated with 1 U Eco-Ung as above. When the repair of U or 8-oxoG was studied, the reaction mixture was supplemented with 400 nM UNG or OGG1, respectively. The reaction mixture was incubated for 20 min at 25 °C and analyzed by 20% denaturing PAGE.

3. Results

dRPase activity can be revealed with 3′-labeled nicked abasic ODN substrates, which were prepared by end-filling of 5′-overhanging ODN duplexes with ^{32}P-labeled dATP and the treatment of the duplex with Ung and APE1. As the resulting dRP site is unstable in nucleophilic buffers and is degraded during migration through Tris-containing gels, the products were stabilized by $NaBH_4$ reduction immediately after the dRPase reaction. Under these conditions, β-elimination of dRP leads to a product with a slightly higher mobility (the bottom arrow in Fig. 3A) compared to the dRP-containing substrate (the middle arrow in Fig. 3A). Fig. 3A illustrates that both NEIL1 and NEIL2 possess a dRP-removing activity. This activity was similar in potassium phosphate and Tris–HCl buffers and was not affected by the presence or absence of Mg^{2+} ions in the reaction mixture (data not shown). The dRPase activities of NEIL1 and NEIL2 demonstrated the enzyme concentration and time dependence expected of an enzyme-catalyzed reaction (Fig. 3B and data not shown). The activity of NEIL1 in these experiments appeared higher than that of NEIL2 (Fig. 3B). Both NEIL1 and NEIL2 excised

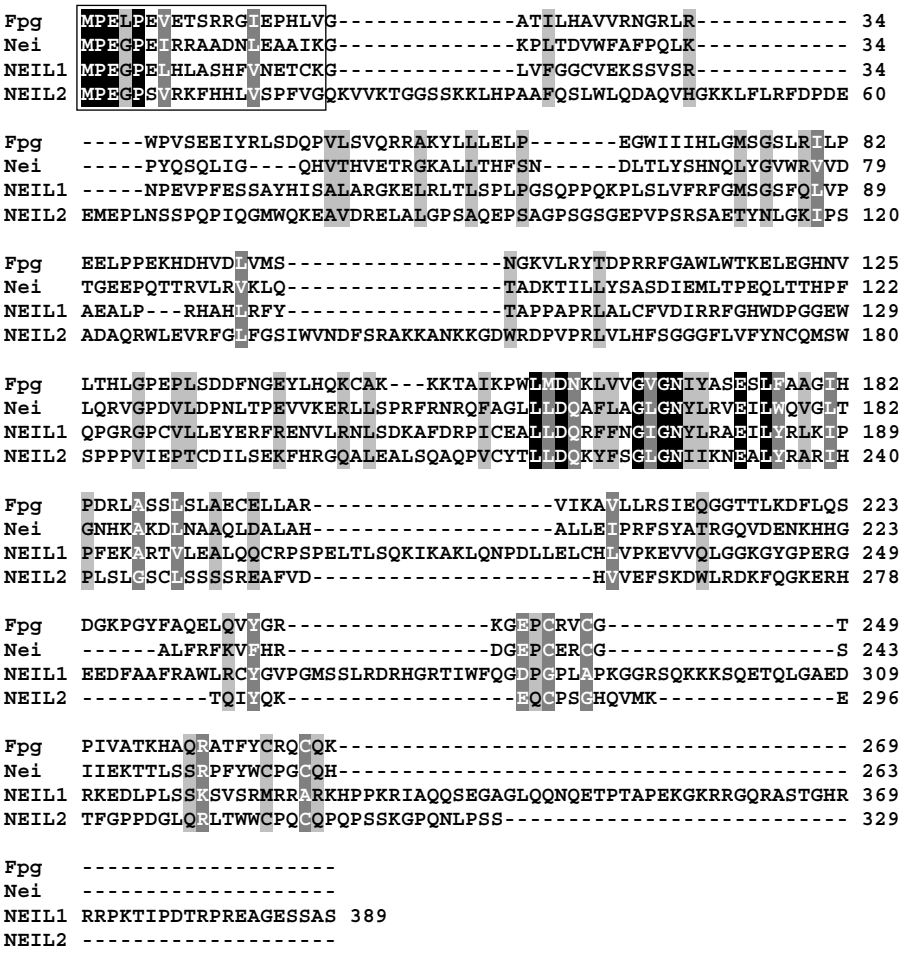

Fig. 2. Alignment of *Escherichia coli* Fpg and Nei with murine NEIL proteins. The N-terminal PE helix inferred from the crystal structure of Fpg, Nei and NEIL1 is boxed. Highlighting indicates absolutely conserved (black with white lettering), highly conserved ($C_n \geqslant 9$; dark gray with white lettering) and conserved ($C_n \geqslant 7$; light gray) positions; conservation numbers are calculated using the AMAS algorithm [48] from the standard Taylor set of physicochemical properties [49]. The alignment was produced by Clustal W 1.82 [50].

dRP with similar efficiency when A, C, or T were placed opposite the lesion, and the excision opposite G was 1.5–2-fold lower (data not shown); Polβ removed dRP equally well from all opposite-base contexts.

To confirm that dRP removal by NEIL1 and NEIL2 proceeds by β-elimination, as in Polβ and Fpg, we have performed the reaction in the presence of $NaBH_4$, which reduces the Schiff base formed between the catalytic nucleophile of dRP lyases and C1′ of the dRP site. Such trapped enzyme–DNA complexes are stable enough to be resolved by regular SDS–PAGE. As can be seen from Fig. 3C, NEIL1 and NEIL2, as well as Fpg and Polβ, formed low-mobility complexes upon incubation with the 3′-labeled dRP-containing substrate and $NaBH_4$, although the extent of crosslinking was rather low due to competing fast reduction of dRP. The molecular masses of the complexes were in the expected order, from the highest (NEIL1, 43.5 kDa of the protein part) to the intermediate (NEIL2 and Polβ, 38.2 kDa and 39.0 kDa, respectively) to the lowest (Fpg, 30.2 kDa). In addition, this experiment shows that the observed dRP lyase activity is not due to a contamination by Fpg or Nei from the *E. coli* expression host, since in this case the mobility of the cross-linked species would correspond to the Fpg–DNA complex.

To compare the efficiency of NEIL1 and NEIL2 as dRPases with the same activity of DNA polymerase β, the best-known mammalian dRPase, we have analyzed steady-state enzyme kinetic for all three enzymes. The results of these experiments are summarized in Table 1. The kinetic data suggest that NEIL1 is as good a dRPase as Polβ, and they both surpassed NEIL2 in their ability to remove dRP from DNA. K_M of NEIL1 was ~5-fold lower than K_M of Polβ, indicating that NEIL1 might bind dRP-containing substrate more tightly; on the other hand, Polβ processed the substrate ~5-fold faster than did NEIL1, resulting in nearly equal specificity constants for both enzymes. NEIL2 had an intermediate catalytic constant and the poorest binding of all three mammalian dRP lyases.

In regular BER, dRP is mainly removed by Polβ dRPase after the insertion of the correct nucleotide by the polymerase activity of Polβ (Fig. 1, steps c and d). We have used a dRPase-deficient Polβ mutant K35A/K68A/K72A (mPolβ), capable of nucleotide insertion but not dRP removal, to inquire whether the dRPase activities of NEIL proteins prefer the substrate with the "hanging" dRP (IV in Fig. 1) to a dRP substrate before the repair DNA synthesis (III in Fig. 1). As Fig. 4 demonstrates, the hanging dRP was indeed a better substrate for both NEIL1 and NEIL2 dRPase activity.

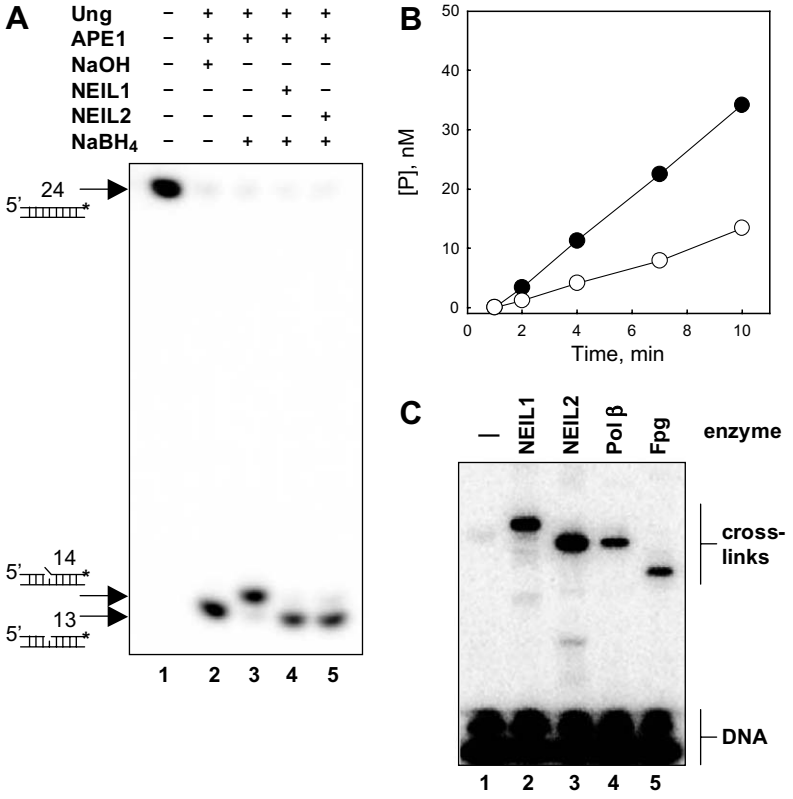

Fig. 3. dRPase activity of NEIL1 and NEIL2. A, Cleavage of a dRP site by NEIL1 and NEIL2. 3′-labeled substrate (20 nM) was treated with 40 nM NEIL1 or 70 nM NEIL2 for 10 min. Lane 1, U-containing ODN; lanes 2–5, dRP-containing ODN treated with alkali (lane 2), NEIL1 (lane 4) or NEIL2 (lane 5). In lanes 3–5, the dRP-containing ODN was stabilized with $NaBH_4$ to prevent its degradation during electrophoresis. Arrows left to the panels indicate positions of the respective ODN species after PAGE. B, Time course of dRP excision by NEIL1 (*filled circles*) and NEIL2 (*open circles*). The concentration of the substrate was 100 nM and of both enzymes, 10 nM. C, Crosslinking of dRP lyases to a dRP-containing substrate (40 nM) stabilized by $NaBH_4$: lane 1, no enzyme; lane 2, NEIL1 (0.9 μM); lane 3, NEIL2 (1.8 μM); lane 4, Polβ (1.8 μM); lane 5, Fpg (0.9 μM).

Table 1
Kinetic parameters of dRPase activity of NEIL1, NEIL2, and Polβ

	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M ($\mu M^{-1}\ min^{-1}$)
NEIL1	0.21 ± 0.03	0.65 ± 0.04	3.1
NEIL2	2.2 ± 0.7	1.6 ± 0.1	0.74
Polβ	1.0 ± 0.1	3.0 ± 0.1	3.0

The experiments with individual enzymes suggest that NEIL1 and NEIL2 possess a dRPase activity and could substitute for Polβ dRPase in BER. To analyze the proficiency of NEIL1 and NEIL2 dRPase in a multienzyme BER process, we have reconstituted the base-excision, AP site-incision, gap-filling and dRP-excision stages of BER using mammalian enzymes (UNG, OGG1, APE1, Polβ and NEIL1 or NEIL2) and ODN substrates containing three widely encountered DNA lesions: U, AP site or 8-oxoG. Fig. 5A shows that when a U-containing DNA is processed by the joint action of UNG and APE1 endonuclease, gap-filling and dRP elimination by a fully functional Polβ generates a high percentage of nicks in DNA subject to further ligation (Fig. 5A, lanes 1–3). If mPolβ was used, the insertion step was as efficient as with wild-type Polβ, but the fraction of ligatable nicks was low (Fig. 5A, lanes 4–6; see the figure legend for definition of the fraction of ligatable nicks), presumably due to the hanging dRP moiety left unrepaired and interfering with DNA ligase. Both NEIL1 (Fig. 5A, lanes 7–9) and NEIL2 (Fig. 5A, lanes 10–12) restored

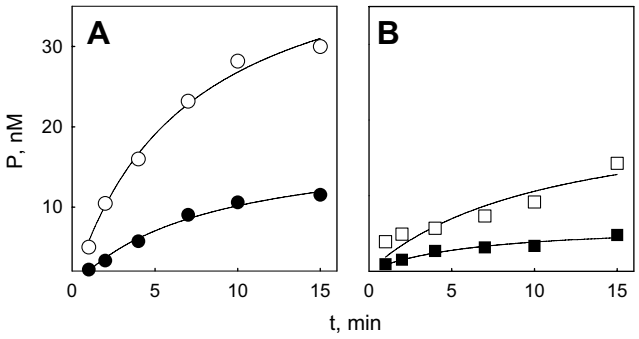

Fig. 4. dRPase activity of NEIL1 and NEIL2 on substrates mimicking post-incision and post-insertion BER intermediates. Panel A: NEIL1 (1 nM). Panel B: NEIL2 (5 nM). *Filled* symbols indicate the control dRPase reactions performed in the presence of 40 nM mPolβ; *open* symbols indicate the dRPase reactions performed after pre-incubation of the dRP substrate with 40 nM mPolβ and 1 mM dGTP to allow insertion of a single nucleotide but not dRP excision by Polβ. Concentration of the substrate was 100 nM.

the ligation efficiency, indicating that they could rescue BER of U lesions driven by a dRP-deficient Polβ. The proficiency of NEIL1 in the full BER was higher compared with NEIL2, in agreement with the kinetic parameters (Table 1). NEIL1, but not NEIL2, alone had weak activity against U in an U:C mispair (Fig. 5A, lane 14), confirming the recent literature reports

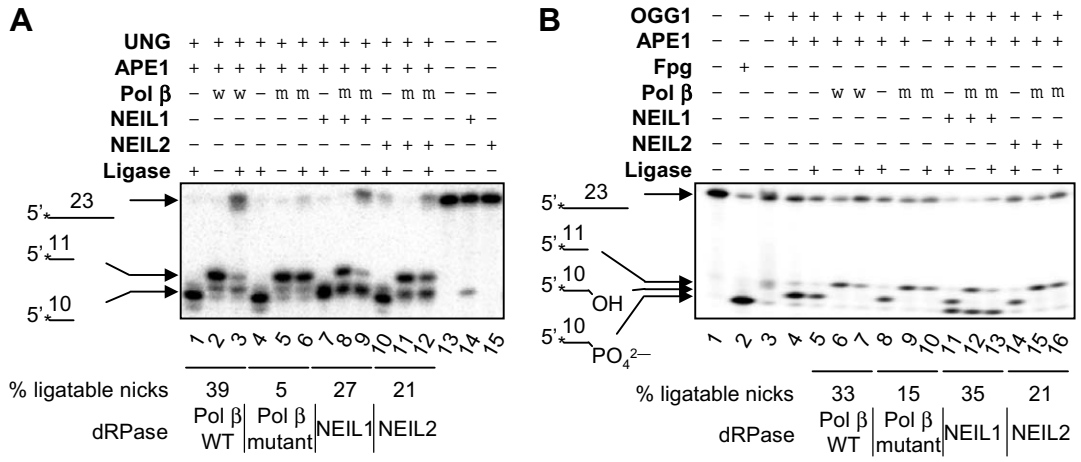

Fig. 5. Reconstitution of BER with NEIL1 and NEIL2 dRPases. (A) 5′-labeled uracil-containing substrate. (B) 5′-labeled 8-oxoG-containing substrate. Constituents of the reaction mixture are shown in the headers; see Section 2 for the reaction conditions. Arrows left to the panels indicate positions of the respective ODN species after PAGE. Percentage of ligatabe nicks is calculated as the percentage of the 23-mer ODN in the full reaction mixture (lanes 3, 6, 9 and 12 in Panel A; lanes 7, 10, 13 and 16 in Panel B, products of the combined β/δ elimination were not included as nicked products since they cannot be extended by Polβ and do not contribute to the religated DNA) minus the percentage of 23-mer DNA in the reaction lacking DNA ligase (lanes 2, 5, 8 and 11 in Panel A; lanes 6, 9, 12 and 15 in Panel B).

[21,28]. This activity obviously did not interfere with further lesion processing by downstream BER enzymes.

We have also reconstituted the repair of AP sites pre-formed in DNA. No major difference from the repair of U was observed, except that the fraction of ligatable nicks was higher for all enzymes (72% for Polβ, 10% for mPolβ, 74% for NEIL1, and 32% for NEIL2; data not shown).

The effect of NEIL1 and NEIL2 with 8-oxoG-containing substrate was not as pronounced due to higher residual repair supported by mPolβ, which might be due to partial removal of the nascent AP site through the combined action of OGG1 (producing α,β-unsaturated 3′-terminal aldehyde) and APE1 (removing this product with formation of a single-nucleotide gap in DNA). Nevertheless, both NEIL proteins could clearly restore the ligation efficiency at dRP sites (Fig. 5B). In the case of NEIL1, the full BER cycle was of lower efficiency due to formation of a β/δ elimination product by the combined action of OGG1 and NEIL1, as recently reported [29]. Such reaction produces a 3′-terminal phosphate residue, which is poorly removed by APE1 (Fig. 5B, the lowermost band in lanes 11–13). When this competing reaction was accounted for and the percentage of ligatable nicks calculated, the effect of NEIL1 was more pronounced than that of NEIL2, as was observed with the repair of U and AP site.

4. Discussion

Removal of a dRP moiety is a critical reaction in eukaryotic BER, defining the dichotomy between single-nucleotide and long-patch repair. If dRP can be removed, the repair synthesis involves insertion of one nucleotide by Polβ and ligation by DNA ligase III/XRCC1 [30]. Otherwise the immediate ligation is impossible and, after insertion of one nucleotide by Polβ [31], a polymerase switch occurs, with further DNA synthesis catalyzed by DNA polymerase δ or ε, promoting a displacement of the downstream DNA strand. The displaced flap structure is later cropped by FEN1 endonuclease, with the resulting nick ligated by DNA ligase I [30]. The two subpathways of BER seem to have different roles. Most notably, disabling single-nucleotide BER in Polβ-null mouse embryonic fibroblasts renders them hypersensitive to DNA-methylating agents, an effect that can be rescued by reinstatement of dRPase but not polymerase activity of Polβ [7] despite long-patch BER is restored in the latter case [11]. Single-nucleotide BER accounts for most of the repair of U [32,33], 8-oxoG [34–36], and thymine glycols [37], whereas both subpathways contribute into the repair of natural AP sites, hypoxanthine and 1,N^6-ethenoadenine [30,35,38].

The ability to carry out single-nucleotide BER is clearly important for cellular genome protection. Removal of dRP is a rate-limiting step in this process [3], representing the most convenient point for the regulation of the entire BER pathway. Unsurprisingly, Polβ is not the only cellular dRPase; several eukaryotic DNA polymerases (γ, ι and λ) have been found to possess this activity [12–16]. BER of 5-hydroxymethyluracil initiated by DNA glycosylase SMUG1 was drastically decreased by additional immunodepletion of DNA polymerase λ in extracts of Polβ-deficient mouse embryo fibroblasts [39]; however, the role of DNA polymerase λ in the repair of other lesions has not been addressed. Unknown proteins of 30–40 kDa are photocrosslinked to substrates imitating dRP in mouse cell extracts [40,41]. Here, we show that two other DNA repair enzymes, NEIL1 and NEIL2, are functional dRPases *in vitro*, with their kinetic parameters comparable to those of Polβ dRPase, and that they can replace Polβ dRPase in a reconstituted BER system.

The *in vivo* functions of mammalian NEIL proteins, discovered in the past five years [18–23], are not entirely clear. DNA glycosylase activity of NEIL1 and NEIL2 against several oxidatively damaged nucleobases have been confirmed [18–23,25,42–45]. However, it generally overlaps with other known mammalian DNA glycosylases, so NEILs have been regarded as back-up enzymes that become important when the major glycosylases are absent [21,46]. Down-regulation of NEIL1 by RNA interference sensitize cells to low-dose radiation

[23], which was attributed to the ability of NEIL1 to excise formamidopyrimidine derivative of adenine and 5*S*,6*R* stereoisomer of thymine glycol. NEIL2 uniquely prefers bubble DNA substrates, suggesting its possible role in DNA repair connected with transcription or replication [42]. Unexpectedly, recently produced NEIL1 knockout mice show no increased DNA damage or carcinogenesis, but suffer from a syndrome apparently related to dysregulation of fat or sugar metabolism [47]. This observation implies that either NEIL1 works in metabolic pathways beyond DNA repair, or that its repair role can be essential in specific organs or tissues, perhaps in adipocytes or brain. Clearly, there is no a priori reason to believe that the functions of NEILs *in vivo* are limited to their glycosylase activity.

As a rule, dRPase activity is fairly low in Polβ-deficient mouse embryonic fibroblasts [7,8] or in the cells with Polβ knocked down by RNA interference (DOZ, unpublished), most likely reflecting the relative abundance of Polβ and other dRPases in this cell line. Still, some residual dRPase activity has been observed in these cells [11]. In addition, other cell types could be less dependent on Polβ as their major dRPase, or other dRPases could act in the repair of specific lesions or at specific points of the cell cycle. Whether NEIL proteins could indeed account for the residual dRPase activity in the absence of Polβ, or could manifest their dRPase activity in some particular *in vivo* systems, is a question warranting further investigation.

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