Psoralen-induced DNA adducts are substrates for the base excision repair pathway in human cells

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ABSTRACT

Interstrand cross-link (ICL) is a covalent modification of both strands of DNA, which prevents DNA strand separation during transcription and replication. Upon photoactivation 8-methoxypsoralen (8-MOP+UVA) alkylates both strands of DNA duplex at the 5,6-double bond of thymidines, generating monoadducts (MAs) and ICLs. It was thought that bulky DNA lesions such as MAs are eliminated only in the nucleotide excision repair pathway. Instead, non-bulky DNA lesions are substrates for DNA glycosylases and AP endonucleases which initiate the base excision repair (BER) pathway. Here we examined whether BER might be involved in the removal of psoralen–DNA photoadducts. The results show that in human cells DNA glycosylase NEIL1 excises the MAs in duplex DNA, subsequently the apurinic/apyrimidinic endonuclease 1, APE1, removes the 3′-phosphate residue at single-strand break generated by NEIL1. The apparent kinetic parameters suggest that NEIL1 excises MAs with high efficiency. Consistent with these results HeLa cells lacking APE1 and/or NEIL1 become hypersensitive to 8-MOP+UVA exposure. Furthermore, we demonstrate that bacterial homologues of NEIL1, the Fpg and Nei proteins, also excise MAs. New substrate specificity of the Fpg/Nei protein family provides an alternative repair pathway for ICLs and bulky DNA damage.

INTRODUCTION

Interstrand cross-links (ICLs) are highly lethal DNA lesions which block DNA transcription and replication by preventing strand separation. Due to their high cytotoxicity, ICL-inducing agents like mitomycin C (MMC), cisplatin and psoralens are widely used against hyperplastic diseases such as cancer and psoriasis. Furanocoumarins (psoralens) are naturally occurring secondary metabolites in plants, including many fruits and vegetables. Among several ICL-inducing agents, psoralens require UVA-photoactivation following DNA intercalation to chemically react with DNA. 8-methoxypsoralen (8-MOP) is a planar, tricyclic compound which intercalates into DNA duplex at pyrimidines, preferentially at 5′-TpA sites. Upon photoactivation, 8-MOP primarily photoalkylates DNA by cycloaddition to the 5,6-double bond of a thymidine, generating two types of monoadducts (MA) with either the 4′,5′-double bond of the furan (MAf) or the 3,4-double bond of the pyrone (MAP) side of the psoralen (1). A unique property of psoralen photochemistry is that the absorption of a second photon by the MAf leads to formation of a pyrene side 5,6-double bond adduct with a flanking thymine in the complementary strand, thus generating an ICL (2). Although the yield of psoralen MAs to pyrimidine bases is 3-fold higher than that of ICLs, the latter class of damage appears to have more severe biological effect (3).

Genetic and biochemical data indicate that elimination of ICLs is mainly linked to DNA replication and involves several linear repair pathways including structure-specific endonucleases, proteins required for homologous recombination-mediated double-strand break (DSB) repair and error-prone translesion DNA polymerases (4–9). The thirteen FANC proteins, mutated in the Fanconi anaemia cancer-prone syndrome, participate in coordination of excision repair of ICLs in order to reconstitute the genetic material with high fidelity. However, at present, the mechanism of coordination and biochemical activities of the FANC proteins involved in the excision of ICLs remains poorly defined. More importantly, it was believed that bulky DNA lesions such as thymine–psoralen adducts can be eliminated only by the nucleotide excision repair (NER) pathway (10). However, with the exception of XPF/ERCC1-deficient cells, cells lacking critical NER components only show a moderate sensitivity to ICL-inducing agents exposure which induces both MA and ICL (11). These observations hint us at the existence of an alternative repair pathway for ICLs.

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NER-independent repair pathway for ICLs and/or bulky DNA adducts.

Non-bulky endogenous oxidative DNA lesions generated by reactive oxygen species (ROS) are substrates for two overlapping pathways: base excision repair (BER) and nucleotide incision repair (NIR). The BER pathway is initiated by a DNA glycosylase cleaving the N-glycosyl bond between the abnormally base and deoxyribose, leaving either an abasic site or a DNA single-strand break which in turn is repaired by an apurinic/apyrimidinic (AP) endonuclease (12). Interestingly, it has been shown that NEIL1 initiates base excision repair of adenine and NTH1 (21,27). Recently, it has been demonstrated that NEIL1 initiates base excision repair of adenine-30-blocking phosphates (14–17). Alternatively, in the NIR pathway, the major human AP endonuclease 1, APE1, incises oxidatively damaged DNA in a DNA glycosylase-independent manner (18). These two pathways can work in concert to cleanse genomic DNA from 7,8-dihydro-8-oxoguanine (8oxoG) residues, one of the major ROS-induced DNA lesions (19). Previously, it was shown that antisense APE1-RNA human cell transfectants exhibited hypersensitivity to MMC, suggesting a potential involvement of APE1 in ICLs repair (20). Furthermore, similar to FA cells, Ape1-depleted cells were sensitive to hyperoxia. APE1 is involved in both BER and NIR suggesting that these pathways may be involved in repair of ICLs.

Human homologues of the Escherichia coli oxidized base-specific DNA glycosylase endonuclease VIII (Nei) were identified by database search of the genome and named NEIL1, 2 and 3 (human Nei-like proteins 1, 2 and 3) (16,21). The Nei-like proteins share significant sequence similitude with the Fpg protein, an 8oxoG-DNA glycosylase found in E. coli. Fpg/Nei/NEIL are structurally and catalytically a distinct family of bi-functional DNA glycosylases endowed with an AP lyase activity that incises DNA at abasic sites by a b,δ-elimination mechanism and leaving single-strand break carrying a phosphate residue at the 3' and 5'-terminus (22,23). Escherichia coli mutants deficient in both DNA glycosylases, endonuclease III (nth) and nei show spontaneous mutator phenotype and are hypersensitive to the lethal effects of oxidizing agents (24,25). Moreover, mouse embryonic stem cell lines deficient in NEIL1 protein were twice more sensitive than control cells to low doses of γ-irradiation (26).

Indeed, NEIL1 initiates base excision repair of adenine-derivative formamidopyrimidines and 5S,6R stereoisomers of thymine glycol (Tg) which are not excised by OGG1 and NTH1 (21,27). Recently, it has been demonstrated that NEIL1 can excise an oxidized base located in the proximity to single-strand break suggesting its involvement in repair of clustered DNA lesions induced by ionizing radiation (28,29). Interestingly, S phase specific expression of NEIL1 and excision of base lesions in single-stranded and bubble-structured DNA substrates point to its possible involvement in replication-dependent repair (21,30).

The purpose of this study was to search for alternative repair pathway(s) involved in removal of ICLs and bulky psoralen–DNA photoadducts. Using a short cross-linked oligonucleotide duplex, we demonstrated that human DNA glycosylase and AP endonuclease initiate alternative repair pathway for bulky psoralen–DNA photoadducts which sought to be substrates for the NIR machinery. The potential biological importance of these findings is discussed.

**MATERIALS AND METHODS**

**Reagents and oligonucleotides**

Chemical reagents including 8-MOP were purchased from Sigma-Aldrich. All oligonucleotides were purchased from Eurogentec (Seraing, Belgium), including regular oligonucleotides, siRNAs and those containing Uracil, 5,6-dihydrouracil (DHU), Tg and 3-terminal phosphate (3'-P). A 21-mer oligonucleotide GFP1, d(GCTCTCGTCTGXAACCCGAAG), where X is either T, U or Tg and complementary ones GFP2, d(GCTCTTCGTTGACAGACCCGAG) and GFP3, d(TTTCGGTTCAGCAGTAGAC) were hybridized to obtain duplexes referred to as GFP1-2 and GFP1-3, respectively (Figure 1C). These sequence contexts were previously used to study the repair of psoralen-induced ICLs in human cells (31).

Another oligonucleotides were used to measure 3'-phosphatase activity: 3'-Exo10, d(TGACTGCTATAP), and complementary 30-mer d(ATGCCATCTGCTACATGCGTGACTGCA). Classic AP site substrate was prepared by treating uracil containing oligonucleotide duplex, we demonstrated that human APE1-depleted cells were sensitive to hyperoxia. APE1 was involved in both BER and NIR suggesting that these pathways may be involved in repair of ICLs.

**Bacterial strains and proteins**

*Escherichia coli* strains AB 1157 (leuB6 thr-1 Δ(gpr-proA2) hisG4 argE3 lacY1 gaIK2 ara-14 nth-1 xyl-5 thi-1 tss-33 rpsL31 supE44 rac) (WT) and its isogenic derivatives BH20 (fgp::kanR) and BH110 (info::kanR [Δ(xth-pncA)90 X::Tn10]) were from the laboratory stock. DNA glycosylase deficient E. coli strains were constructed by insertional mutagenesis as described (33). Briefly, to construct fgp nei double mutant, the spectinomycine resistance cassette (SpcR) was inserted into NEI gene and transferred to the *E. coli* BH20 chromosome by recombination. Cells acquiring the selectable marker were selected and the presence of SpcR gene insertion was further confirmed by PCR of the genomic DNA. Single clone containing inactivated nei::SpcR and fgp::kanR genes (MS2000) was chosen for further work. Strains AB2480 (isogenic to AB1157 except uvrA6 recA13) and SW2-8 (isogenic to BW35 (KL16) except nei::camR) were gifts from Dharmacon (Perbio Science, France).
Dr A. K. McCullough (Oregon Health & Science University, Portland, OR) and Dr S. S. Wallace (University of Vermont, Burlington, VT), respectively.

T4 DNA ligase was purchased from Roche Diagnostics GmbH. The purified *E. coli* Ung, Nfo, Fpg, Nth, AlkA and human OGG1, ANPG and NTH1 proteins were from laboratory stock. Human Ape1 protein was expressed and purified from *E. coli* BH110 (DE3) strain to avoid cross-contamination of bacterial AP endonucleases as described (34). The expression vectors phNEIL1 and phNEIL2 (27), the purified *E. coli* Nei protein (35) and human DNA polymerase β (36) were generously provided by Dr Hiroshi Ide (Hiroshima University, Japan), Dr Dmitry Zharkov (ICBFM, Novosibirsk, Russia) and Dr Grigory Dianov (MRC, Harwell, Oxfordshire, UK), respectively. The full-length native NEIL1 and NEIL2 were purified as described previously (27).

**Preparation of psoralen mono- and diadducts**

Five microgram of plasmid DNA pUC18 or 10 pmol of 32P-labelled GFP1-2 were incubated for 15 min in the dark with 0.1 mM 8-MOP in 50 µl of 100 mM Tris-HCl, pH 7.5, 5 mM EDTA and 50 mM NaCl, then irradiated at 365 nm and 240 kJ/m² at room temperature. Plasmid DNA was purified by ethanol precipitation and the presence of ICLs was verified by denaturing agarose gel electrophoresis. GFP1-2 was desalted by spin-down columns filled with water equilibrated Sephadex G-50. Cross-linked and noncross-linked oligonucleotides were separated by denaturing 20% PAGE. The oligonucleotides were eluted from the gel strips in 2 M LiClO₄ and then acetone precipitated. To obtain MAP residue, the purified cross-linked GFP1-2 was treated with hot alkali (37).

**DNA glycosylase assays**

The standard reaction mixture (20 µl) for DNA glycosylase activity contained either 0.1 µg of pUC19 or 10 nM of 5-[^32]P-labelled GFP1-2 were incubated for 15 min in the dark with 0.1 mM 8-MOP in 50 µl of 100 mM Tris-HCl, pH 7.5, 5 mM EDTA and 50 mM NaCl, then irradiated at 365 nm and 240 kJ/m² at room temperature. Plasmid DNA was purified by ethanol precipitation and the presence of ICLs was verified by denaturing agarose gel electrophoresis. GFP1-2 was desalted by spin-down columns filled with water equilibrated Sephadex G-50. Cross-linked and noncross-linked oligonucleotides were separated by denaturing 20% PAGE. The oligonucleotides were eluted from the gel strips in 2 M LiClO₄ and then acetone precipitated. To obtain MAP residue, the purified cross-linked GFP1-2 was treated with hot alkali (37).

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**Formation of enzyme–DNA covalent complexes**

The OGG1, NTH1 and NEIL1 proteins (200 nM) were incubated with 0.2 pmol of 5-MOP+UVA-treated 5-[^32]P]-labelled GFP1-2 at 37°C for 30 min in 20 µl of the standard reaction mixture but in the presence of 50 mM NaBH₄ (a 2 M NaBH₄ stock solution in water was prepared immediately prior to use) or 100 mM NaCl as a control. The reaction was stopped by adding 5 µl of buffer containing 10% SDS, 30% glycerol, 25% 2-mercaptoethanol, 0.1% bromophenol blue and 300 mM Tris-HCl, pH 6.8 and heating 10 min at 60°C. The reaction products were separated by 15% SDS-PAGE and analysed as described above.

**Cell culture, drug treatment and survival assay**

All strains of *E. coli* were grown with shaking at 37°C to 3 x 10⁸ cells/ml in LB medium. Cells were centrifuged and resuspended in M9 medium at a density 5 x 10⁸ cells/ml before irradiation. When appropriate, 8-MOP was added to a final concentration of 5 µM, and the suspension was allowed to stand on ice for 10 min in the dark before irradiation. Samples were irradiated at room temperature with HPW125 Philips lamp with a pyrex water filter. The fluence through the sample was 1 mW/cm². All experiments were carried out at least three times.

HeLa cells were routinely grown at 37°C in 5% CO₂ in Dulbecco minimal essential medium supplemented with 10% foetal calf serum, 2 mM glutamine, 100 U/ml of penicillin and 100 mg/ml of streptomycin. To decrease the target protein level, HeLa cells were plated in 6-well plates at the concentration of 180 000 cells/well and 24 h later were transfected with 100 nM of appropriate siRNA using Oligofectamine (Invitrogen) as described (38).

Cells were seeded into 96-well plate at the concentration of 10⁴ cells/well in complete medium 72 h after siRNA transfection. ICL induction by photoactivated psoralen was achieved by incubating cells with 10 µM of 8-MOP for 20 min followed by exposure to 10 kJ/m² UVA. Afterwards, cells were cultivated at 37°C in complete medium for increasing periods of time. Cell viability was assessed using Cell Proliferation Kit II (XTT) (Roche Diagnostics GmbH) according to the manufacturer instructions.

For immunoblotting cells were sonicated in Laemmli loading buffer. Total proteins were quantified by Bradford assay, and separated by SDS-PAGE, then electroblotted
onto a nitrocellulose membrane (BA85, Schleicher & Schull). The membranes were saturated in 5% non-fat dry milk in PBS for 1 h at room temperature before adding primary antibodies for overnight at 4°C. The following antibodies were used: NEIL1 (1:600; Abcam), actin (1:500), APE1 (1:5000; Eurogentec, Belgium). Binding of the primary antibodies was detected with HRP-coupled secondary antibodies followed by enhanced chemiluminescence detection (GE Healthcare) and visualized with a GeneGnome gel documentation system (Syngene, MD, USA).

RESULTS

Role of the base excision repair proteins in the cellular response to DNA cross-linking agents

APE1−/− cells are sensitive to MMC, however, this cross-linking agent generates a variety of oxidative and non-oxidative DNA lesions and only 10% of them are ICLs (6). Consequently, to examine the potential involvement of APE1 in ICLs processing, we used exposure to photoactivated 8-MOP that generates distinct, well-characterized and easily detectable DNA adducts. The sensitivity of HeLa cells with reduced expression of APE1 and FANCD2 towards several genotoxins was examined. In agreement with the previous observations, HeLa cells transfected with Ape1-specific siRNA were highly sensitive to ionizing radiation (IR) and MMC but not to UVC treatments (data not shown). Strikingly, the cells with decreased Ape1 level were also sensitive to 8-MOP+UVA exposure, like cells with reduced expression of FANCD2 protein, suggesting a potential involvement of Ape1 in the processing of psoralen–DNA adducts (Figure 1A).

The sensitivity of APE1-depleted HeLa cells to cross-linking agents suggest that APE1-catalysed activities may remove psoralen-DNA adducts and thus perform function similar to that of the structure-specific endonuclease XPF-ERCC1 (8). In fact, in the NER pathway APE1 can incise the DNA sugar-phosphate backbone 5’ next to bulky lesions such as 3,N4-benzetheno-2’-deoxynucleotides (39), thus providing a back-up function to NER pathway. Furthermore, in the BER pathway, APE1 acts downstream of various DNA glycosylases to eliminate genotoxic repair intermediates. Thus depletion of APE1 removes the major AP and NIR endonuclease and 3’-repair diesterase activities in human cells, severely disabling two repair pathways. The first approach was to study whether the purified BER proteins cleave DNA cross-links and for this purpose 8-MOP+UVA treated supercoiled (sc) plasmid DNA was incubated with APE1 and human DNA glycosylases NTH1, NEIL1 and OGG1. Upon cleavage at the site of damage by a DNA repair enzyme, the sc form is converted to an open circular (oc) form and these two forms are identified by electrophoresis in agarose gel (40). As shown in Figure 1B, only NEIL1 converts the sc form to oc one, indicating that it specifically recognizes and incises photoactivated psoralen–DNA adducts (lane 7).

Oxidative DNA glycosylase, NEIL1, excises psoralen-thymine MAs present in duplex DNA

To further examine whether NEIL1 excises MAs or ICLs or both, the short 21-mer 5’-[32P]- and 3’-[32P]-dCMP-labelled oligonucleotide duplexes, GFP12, were treated with 8-MOP+UVA and the cross-linked oligonucleotides were separated from non-cross-linked ones by denaturing
PAGE and used as DNA substrates. Interestingly, NEIL1 cleaves only non-cross-linked GFP1·C1 (Figure 2A, lanes 4, 8 and 12). The 5′ and 3′-labelled cleavage fragments migrating at position corresponding to an 11-mer (lanes 4 and 8) and 10-mer (lane 12), respectively, indicate excision of a psoralen–thymine MA at the position 12 of the 21-mer oligonucleotide. Consistent with the established mechanism of action of bi-functional DNA glycosylases/AP lyases, NEIL1 generates a Schiff base intermediate that can be characterized by the covalent trapping of the enzyme–substrate complex by reduction with NaBH₄ (Figure 2B, lane 4) (41). To characterize the substrate specificity of NEIL1, an oligonucleotide containing single MAp was constructed by incubating cross-linked GFP1·C1 under hot-alkali conditions (42). As expected, base-catalysed reversal of the cross-link yields two closely migrating DNA fragments, the ‘faster migrating’ native and ‘slow migrating’ MAp-containing oligonucleotides (Figure 3A, lane 5). Appearance of an 11-mer fragment (lane 6) upon incubation with NEIL1 was accompanied by the loss of MAp-containing fragment indicating that NEIL1 excises MAp residues present in duplex DNA. Furthermore, NEIL1 cleaves GFP1·C1 irradiated at 405 nm suggesting that it also excises MAfs (data not shown).

To further characterize the substrate specificities of the NEIL1 protein, the kinetic constants for the excision of MAp and DHU·C1 were measured. Comparison of the kinetic constants (Table 1) shows that MAp is the preferred substrate for NEIL1 ($K_M = 6.3 \text{nM}$, $k_{cat}/K_M = 3.9 \text{min}^{-1} \mu\text{M}^{-1}$) which is recognized with high specificity.

NEIL1-generated DNA 3′-phosphate termini are removed by APE1

To examine the nature of 3′-termini generated by NEIL1-catalysed excision of MAp, 5′-[32P]-labelled GFP1·C1 duplexes containing an AP site, MAp or Tg residues were used. As shown in Figure 3B, when excising a Tg, AP site and MAp NEIL1 generates 11-mer DNA fragments migrating the same distance a result that is consistent with β,δ-elimination mechanism of action of this enzyme (lanes 3, 5 and 9). In agreement with this observation, DNA cleavage fragments generated by the NEIL1 and E. coli Nei proteins (lanes 8–9) migrate faster than products generated by NTH1 and APE1 containing 3′-α,β-unsaturated aldehyde and 3′-hydroxyl residues, respectively (lanes 2 and 6). Taken together, these results demonstrate that with all DNA substrate tested NEIL1 and Nei generate products carrying a phosphate at the 3′-terminus.

The catalytic rate of human APE1 acting on 3′-phosphoglycolates and 3′-Ps is nearly 200-fold lower than its AP endonuclease function (43). Recently, Wiederhold and colleagues (13) showed that polynucleotide kinase and not APE1 is primarily involved in the NEIL1-initiated BER pathway. To examine whether 3′-Ps are removed by APE1 under reaction conditions used, we incubated 5′-[32P]-labelled 10-mer DNA fragment carrying 3′-P residue with the E. coli Nfo and varying amounts of APE1 proteins at 30 and 37°C. As shown in Figure 4, at 30°C 5 nM APE1 removes more than 50% of 3′-P (lanes 3 and 7) whereas 20 nM APE1 and 5 nM Nfo eliminate all 3′-P residues generating 10-mer fragment containing 3′-hydroxyl with lower mobility as compared

Figure 2. The BER pathway removes psoralen-induced monoadducts.

(A) NEIL1 is active on 8-MOP+UVA-induced MAs. 5′-[32P]-dCMP-labelled GFP1·C1 (lanes 9–12) and 5′-[32P]-labelled GFP2 (lanes 5–8) were treated with 8-MOP+UVA. Cross-linked GFP1·C1 (lanes 1, 2, 5, 6, 9 and 10) was purified from non-cross-linked GFP1·C1 (lanes 3, 4, 7, 8, 11 and 12) and incubated or not with NEIL1. ‘a’ indicates cross-linked oligonucleotide duplex, ‘b’ non-cross-linked oligonucleotide duplex containing single MA. (B) The AP lyase activity of NEIL1 on GFP1·C1 containing psoralen MA. Purified non-cross-linked 5′-[32P]-labelled GFP1·C1 was incubated with 200 nM of OGG1, NTH1 and NEIL1 in presence of NaBH₄ at 37°C for 30 min before separation of the DNA–protein covalent complex and free DNA by 10% SDS-PAGE.
to the substrate DNA (lanes 2, 4, 6 and 8). Addition of 1 mM ATP did not affect activity of enzymes. 3'-Phosphatase activity of APE1 but not that of Nfo is strongly inhibited at 37°C (lanes 10–12 and 14–16).

Interestingly, APE1 at higher protein concentration exhibits significant 3'→5' exonuclease activity generating 9-mer DNA fragment (lanes 4 and 8). These results show that at physiologically relevant protein concentration, APE1 exhibits efficient 3'-phosphatase activity.

In vitro reconstitution of the BER pathway for bulky psoralen–DNA photoadducts

As we showed above, NEIL1-catalysed excision of MA generates a single-strand break with 3'-P terminus which has to be eliminated. Hence, we have reconstituted in vitro the BER pathway for MAps using purified proteins (Figure 5). Incubation of a 21-mer GFP1-3 containing a single MAp in the presence of NEIL1, APE1, DNA polymerase β and [α-32P]-TTP generated a labelled 12-mer DNA fragment (lane 4). The results indicate that APE1 removes 3'-P residues generated by NEIL1 and allows DNA polymerase β to insert one nucleotide. Addition of DNA ligase completes the restoration of the full-length 21-mer GFP1-3 (lane 5). These data demonstrate that 8-MOP-induced MAs are processed by the short-patch BER pathway. In agreement with biochemical data, the siRNA-transfected cells with decreased NEIL1 level were hypersensitive to 8-MOP+UVA exposure (Figure 6). It should be stressed that we did not observe significant additive increase in the sensitivity when cells were depleted for both APE1 and NEIL1. This result indicates that both proteins participate to a same pathway to cope with psoralen-induced DNA damage. These in vitro and in vivo data imply new functions of NEIL1 and APE1 in the removal of DNA adducts generated by cross-linking agents.

Activity of various *E. coli* and human DNA repair proteins on oligodeoxynucleotides containing MAp

To further investigate the excision of psoralen MA in DNA, we examined whether this lesion was a substrate for previously characterized BER enzymes from *E. coli* and human. We challenged a 5'-[32P]-labelled oligonucleotide duplex containing single MAp with a variety of highly purified AP endonucleases and DNA glycosylases. Since not all DNA glycosylases possess AP-nicking activity, the assays were made in the presence of AP endonuclease in order to cleave DNA duplex at the potential abasic sites generated by the base excision. When the various *E. coli* and human enzymes were tested on GFP1/C1 (Figure 7A), only incubation with Fpg, Nei and NEIL1 led to the cleavage of the labelled oligonucleotide at the position of the psoralen–thymine MAp. Interestingly, the excision of MAp by the Nei-like enzymes was more efficient than that of Fpg (lanes 5 and 7 versus 4). Despite being used in excess amount, Nfo, AlkA, Nth, OGG1, NEIL2, APE1, ANPG and NTH1 proteins did not act on GFP1/C1 (lanes 2–3, 6, 8–11 and data not shown).

Photoactivated psoralen sensitivity of DNA glycosylase-deficient *E. coli* mutants

Genetic and biochemical studies of *E. coli* have established that both the RecA-dependent recombination and the NER pathways participate in removal of MAps.
psoralen-induced cross-links (44,45). Subsequently, it was demonstrated that psoralen MAs are removed by UvrABC nuclease in vitro with high efficiency (46). To address the physiological relevance of the Nei and Fpg-catalysed excision of psoralen MAs, we assessed the sensitivity of the *E. coli* mutant strains to 8-MOP+UVA exposure. As shown in Figure 7B, control *E. coli uvrB recA* strain was extremely sensitive to the photoactivated psoralen, whereas *E. coli nei* and *fpg* single and *nei fpg* double mutants were not particularly sensitive to treatment. These results suggest that in bacteria, the NER system is a major pathway for removal of psoralen MAs present in DNA.

**DISCUSSION**

Repair of ICLs and other complex DNA lesions in mammalian cells is linked to replication and required successive involvement of several distinct repair pathways in coordinated manner. At present, the underlying mechanism of this coordination is poorly understood. The results obtained in this study reveal that psoralen–thymine MAs present in duplex DNA are substrate for human oxidative DNA glycosylase, NEIL1. Analysis of the kinetic data demonstrates that NEIL1 can efficiently backup the NER pathway to repair psoralen-induced MAs. It has been shown that NEIL1 catalyses the β,δ-elimination at AP site after oxidized base excision, leaving a 3'-P at the resulting single-strand break (16).

Here, we demonstrated using size-markers generated by Nei, NTH1 and APE1 that NEIL1 in fact generates 3'-P termini while removing the psoralen adduct. Based on the fact that PNK has a much higher 3'-phosphatase activity than APE1, it has been proposed that in mammalian cells 3'-P generated by NEIL1 and NEIL2 is removed by PNK in the AP endonuclease-independent BER pathway (13). Although, human major AP endonuclease is a weak 3'-phosphatase, we demonstrated that at 20 nM protein concentration APE1 efficiently repairs oligonucleotide fragment containing 3'-P residue. In agreement with this result, we have fully reconstituted in vitro the repair of MAs using purified APE1, NEIL1, DNA polymerase β and DNA ligase. Following NEIL1 excision APE1 removes 3'-P residue and allows subsequent gap filling and ligation. Consistent with the biochemical data, HeLa cells lacking APE1 and/or NEIL1 become hypersensitive to 8-MOP+UVA exposure. Thus, we added one arm of the BER pathway to the cellular defence used to cope with the genotoxic effects of cross-linking agents. These results demonstrate that NEIL1 is not simply a backup DNA glycosylase for OGG1, NTH1 and NEIL2 but has distinct substrate specificity towards a specific class of bulky DNA adducts.
NEIL1, a 44 kDa protein, was initially characterized as a DNA glycosylase specific for oxidized, saturated and ring-fragmented bases. Several lines of evidence argue for a biological role of NEIL1 in counteracting oxidative DNA damage. Oxidative stress could transiently increase NEIL1 level in human colon carcinoma cells (47). Analysis of human polymorphic and mutant variants of NEIL1 revealed that their low DNA glycosylase activities and reduced expression may be involved in the pathogenesis in a subset of gastric cancers and increased risk of metabolic syndrome (48, 49). Cells deficient in the Werner syndrome protein (WRN), a member of the RecQ family of DNA helicases, are hypersensitive to psoralen induced ICLs (50, 51). Lately, it has been shown that WRN interacts with and stimulates NEIL1 in excision of oxidative lesions from bubble DNA substrates suggesting that NEIL1–WRN complex participates in the same repair pathway (52). Recently, Vartanian and colleagues (53) generated knockout (KO) mice with a frameshift deletion and insertion in the NEIL1 gene. The steady-state mitochondrial DNA damage and deletions from liver tissues of NEIL1−/− KO mice were significantly increased compared to wild-type controls. The mice develop spontaneously severe obesity, dyslipidemia and fatty liver disease, suggesting that accumulation of spontaneous DNA damage in cells lacking NEIL1 may lead to the diseases associated with the metabolic syndrome. Interestingly, dietary consumption or handling...
of psoralen-containing plants have been shown to cause adverse effects to human health (54). In light of a new function of NEIL1, it is tempting to speculate that NEIL1 could be more beneficial to psoralen-rich diet.

Since human DNA glycosylase can excise bulky MAP, we investigated whether this lesion was a substrate for previously characterized E. coli Nfo, AlkA, Nth, Fpg and Nei and human APE1, OGG1, NTH1, NEIL2 and ANPG proteins. The results show that in addition to NEIL1, the bacterial Fpg and Nei proteins can also excise psoralen MAP when present in duplex oligonucleotides. In E. coli, psoralen MAs are good substrates for UvrABC nuclease (46) however both NER and homologous recombination pathways are required to remove the psoralen-induced ICLs (55). Evolutionary conservation of the new substrate specificity in bacterial Nei-like DNA glycosylases suggests its possible biological role. Therefore, we examined whether in E. coli Fpg/Nei-catalysed excision of MAP provides an alternative pathway to classic NER. Surprisingly, E. coli DNA glycosylase-deficient mutants, in contrast to uvrA recA mutant, were not sensitive to 8-MOP + UVA exposure indicating that in E. coli NER is a major pathway to remove psoralen MAs.

Three-dimensional crystal structures of the Nei–DNA complex and free NEIL1 protein show the same overall fold for both DNA glycosylases (35,56). Both proteins consist of two domains connected by a linker, this module structure giving rise to a DNA-binding cleft between domains. Based on molecular modelling it was suggested that Nei binds to DNA and flips out damaged base in a shallow hydrophobic pocket (35). At present, structural information for Nei and NEIL1 complexes with DNA duplex containing an oxidized base is not yet available. A bulky psoralen MA presents topological constraint for its accommodation in the active sites of Nei-like DNA glycosylases. A 3D crystal structure of the complex of T4 endonuclease V bound to a DNA substrate containing a bulky cyclobutane thymine dimer showed that the enzyme kinks the DNA helix by about 60° and flips out the opposing adenine base complementary to thymine dimer out of the DNA base stack (57). Based on these observations we may hypothesize that the mechanism of lesion recognition by Nei-like enzymes is different from that of well-studied DNA glycosylases. To access the C10 observations we may hypothesize that the mechanism of dimer out of the DNA base stack (57). Based on these insights into the structural basis for clustered and bulky DNA damage recognition by Nei-family DNA glycosylases will have to await further investigations.

In conclusion, although in vitro NEIL1 does not incise DNA containing single ICL, we hypothesized that it may participate in psoralen-induced ICLs removal after XPF-ERCC1-mediated unhooking. Alternatively, but not exclusively, NEIL1/APE1 action could significantly reduce ICLs formation by eliminating MAP residues.

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