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AP endonuclease 1 prevents the extension of a T/G mismatch by DNA polymerase β to prevent mutations in CpGs during base excision repair

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Abstract

Dynamics of DNA methylation and demethylation at CpG clusters are involved in gene regulation. CpG clusters have been identified as hot spots of mutagenesis because of their susceptibility to oxidative DNA damage. Damaged Cs and Gs at CpGs can disrupt a normal DNA methylation pattern through modulation of DNA methylation and demethylation, leading to mutations and deregulation of gene expression. DNA base excision repair (BER) plays a dual role of repairing oxidative DNA damage and mediating an active DNA demethylation pathway on CpG clusters through removal of a T/G mismatch resulting from deamination of a 5mC adjacent to a guanine that can be simultaneously damaged by oxidative stress. However, it remains unknown how BER processes clustered lesions in CpGs and what are the consequences from the repair of these lesions. In this study, we examined BER of an abasic lesion next to a DNA demethylation intermediate, the T/G mismatch in a CpG dinucleotide, and its effect on the integrity of CpGs. Surprisingly, we found that the abasic lesion completely abolished the activity of thymine DNA glycosylase (TDG) for removing the mismatched T. However, we found that APE1 could still efficiently incise the abasic lesion leaving a 3-terminus mismatched T, which was subsequently extended by pol β . This in turn resulted in a C to T transition mutation. Interestingly, we also found that APE1 3'–5' exonuclease activity efficiently removed the mismatched T, thereby preventing pol β extension of the mismatched nucleotide and the resulting mutation. Our results demonstrate a crucial role of APE1 3'–5' exonuclease activity in combating mutations in CpG clusters caused by an intermediate of DNA demethylation during BER.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Keywords

DNA demethylation; Base excision repair; DNA damage; DNA polymerase β ; AP endonuclease 1; CpG islands

1. Introduction

Dynamics of cytosine methylation, i.e., formation of 5-methyl cytosine (5mC) and demethylation of 5mC in CpG islands (CGIs) in the mammalian genome are actively involved in the regulation of gene expression, inactivation of the X chromosome, and gene imprinting, among others [1–4]. It is estimated that only approximately 1–2% of cytosines in the mammalian genome are unmethylated [5–8], whereas 60–90% of CpGs are methylated [1,9]. The DNA methylation pattern regulates gene transcription by facilitating or blocking access of transcription factors to gene promoter or transcribed regions directly [10,11], or by modulating the recruitment of methyl CpG binding proteins [12–15], as well as by altering histone modifications and chromatin structures [16–18]. A normal DNA methylation pattern is essential for maintaining the homeostasis of gene expression and cellular function, whereas an aberrant DNA methylation pattern is associated with the onset and progression of many diseases [19–21]. It has been found that hyper-methylation of CpGs on tumor suppressor genes (TSGs) [22,23] and hypomethylation of CpGs on oncogenes result in deregulation of expression of the genes leading to the development of cancer [4].

5mC can be removed by a process called DNA demethylation, which can occur either passively or actively. Passive DNA demethylation results from the failure of DNA methyltransferases to methylate a cytosine during DNA replication and cell division [2]. Passive DNA demethylation may also result from the loss or substitution of cytosines by DNA damage and repair in the context of CpGs. Active DNA demethylation is mediated by sequential enzymatic reactions that can occur independent of DNA replication and cell division [2,3,24,25]. Removal of a 5mC by active DNA demethylation is initiated by modifications of the nucleotide through several types of enzymatic reactions, including hydroxylation, deamination and oxidation that convert the 5mC into a modified base or base lesion that is recognized and cleaved by DNA glycosylases [2,3]. This subsequently leads to replacement of the 5mC through the DNA base excision repair (BER) pathway [2,3,25]. It has been shown that BER-mediated active DNA demethylation is accomplished through several pathways depending on the type of modifications of 5mC that are removed by different types of DNA glycosylases [2,3,24,26]. One of the pathways is initiated by a direct deamination of 5mC by activation-induced cytidine deaminase (AID) that converts a 5mC to a thymine, resulting in a T/G mismatch [27]. The mismatched T can be subsequently excised by thymine DNA glycosylase (TDG) [25,28–30], leaving an abasic site that is then subjected to BER. This ultimately leads to replacement of the 5mC with an unmethylated C [2]. Another BER-mediated DNA demethylation pathway is initiated by single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1) that removes a 5-formyluracil generated from oxidation of a 5mC by a family of enzymes called Ten Eleven Translocation (TET), a methylcytosine dioxygenase [31–33], or from oxidative DNA damage induced by

hydroxyl radicals [34,35]. Thus, in mammalian cells, DNA base damage and BER are strategically used as a mechanism for both passive and active DNA demethylation [26].

While the cytosine in a CpG dinucleotide is a substrate for DNA methylation and demethylation, the neighboring 3'-guanine is a hot spot of oxidative DNA damage. As the most abundant form of oxidative DNA damage in mammalian cells, 8-oxoguanine (8-oxoG) readily accumulates in CpGs and may affect the integrity of CpGs by modulating the production and processing of DNA demethylation intermediates. It has been found that 8-oxoG can cause accumulation of T/G mismatched base pairs when it occurs adjacent to 5mC by inhibiting the removal of the mismatched T [36]. Moreover, when BER of 8-oxoG encounters active DNA demethylation, repair of the lesion may be affected by a DNA demethylation intermediate. This has been supported by a recent study from the Wilson group showing that the efficiency of removal of 8-oxoG by OGG1 was significantly reduced by an adjacent 5'-T/G mismatch, a DNA demethylation intermediate generated from deamination of 5mC in CpGs [36]. This subsequently inhibited the completion of BER. This suggests that a base lesion interferes with an essential step of BER-mediated DNA demethylation, thereby compromising the efficiency and fidelity of DNA demethylation in CpGs. These studies further suggest that BER of oxidative DNA damage in CpGs and DNA demethylation intermediates has to be properly coordinated to maintain the integrity and fidelity of CpGs. However, it remains unknown how the fidelity of CpGs may be maintained by BER in coordinating its dual function in repairing a base lesion as well as in mediating active DNA demethylation. In this study, we explored how an abasic lesion that occurs in a CpG at the 3'-side of a T/G mismatch could affect the integrity of a CpG dinucleotide by modulating the removal of the mismatched T during BER, and how BER may coordinate the removal of the base lesion and maintenance of the fidelity of CpG dinucleotides. For the first time, we have shown that the abasic lesion completely inhibited the removal of its adjacent 5'-mismatched T by TDG leading to accumulation of the mismatched nucleotide. We show that DNA polymerase β (pol β) readily tolerated the mismatched T and efficiently extended the mismatched nucleotide, allowing the sustainment of the T/G mismatch and leading to a C to T transition mutation during BER. Interestingly, we discovered that AP endonuclease 1 (APE1) 3'-5' exonuclease efficiently removed the mismatched T, thereby preventing the mutation. Our results indicate that APE1 3'-5' exonuclease plays a crucial role in maintaining the integrity of CpGs during BER and DNA demethylation. This demonstrates that the coordination between BER enzymes effectively removes a 3'-mismatched T, thus preventing mutations that result from BER and BER-mediated active DNA demethylation. Our study provides new insights into the molecular mechanisms underlying the roles of BER in preventing T/G mismatches and sustaining the integrity of CpGs during BER and active DNA demethylation.

2. Materials and methods

2.1. Materials

Oligonucleotides were synthesized by Integrated DNA Technologies Inc. (Coralville, IA). The radionucleotides [γ - ^{32}P] ATP (6000Ci/mmol) and cordycepin 5'-triphosphate 3'-[α - ^{32}P] (5000Ci/mmol) were purchased from PerkinElmer Inc. (Boston, MA). Micro Bio-Spin 6

chromatography columns were from Bio-Rad (Hercules, CA). T4 polynucleotide kinase (PNK) and terminal deoxynucleotidyl transferase (*TdT*) were from Fermentas (Glen Burnie, MD). Adenosine 5'-triphosphate (ATP) (100 mM) was from USB (Cleveland, Ohio). Purified thymine DNA glycosylase (TDG) was from Enzymax, LLC (Lexington, Kentucky). Purified APE1, pol β , flap endonuclease 1 (FEN1) and DNA ligase I (LIG I) were generous gifts from Dr. Samuel H. Wilson at the Genome Integrity and Structural Biology Laboratory, National Institute of Environmental Health Science/National Institutes of Health (NIEHS), Research Triangle Park, NC. All other reagents were from Thermo Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich (St. Louis, MO).

2.2. Oligonucleotide substrates

An oligonucleotide substrate containing a T/G mismatched base pair adjacent to a G/C matched base pair or a tetrahydrofuran (THF), an abasic site analog, was designed to mimic the intermediates resulting from deamination of a 5-methylcytosine by AID in a CpG dinucleotide with an undamaged G or an abasic lesion (AP site) that substituted G. Substrates containing a T/G mismatch at the upstream primer and a 5'-THF residue at the downstream primer were designed to mimic the BER intermediates containing an oxidized AP site adjacent to a T/G mismatch that is 5'-incised by APE1 opposite to a template C or T. The substrate containing an intact or preincised AP site opposite to a template C and adjacent to a C/G matched base pair was used as the control. Substrates for measuring TDG activity were constructed by annealing the strand containing a T with the template strand containing a G that was base paired with the T at a molar ratio of 1:1.5. Substrates for measuring BER enzymatic activity were constructed by annealing the upstream primer with a 3'-T that mispaired with a template G and the downstream primer with a 5'-THF residue to the template strand at a molar ratio of 1:1:2. The sequences of the oligonucleotide substrates are listed in Supplementary Table S1.

2.3. Measurement of TDG activity in removing a T/G mismatch adjacent to a THF residue, an analogue of an AP site

TDG activity for removing the T from a T/G mismatch adjacent to a THF residue was measured by incubating increasing concentrations of TDG from 50 nM to 70 nM with 25 nM substrate containing a T/G mismatch with or without the THF residue. The activity was examined at 37 °C for 30 min in 10 μ l reaction buffer with 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, and 0.01% Nonidet P-40. Reactions were terminated by transferring to 95 °C for 5 min. The reaction mixture was then treated with 0.1 M NaOH and denatured at 95 °C for 10 min in buffer containing 95% formamide and 10 mM EDTA. Substrates and products were separated by 15% urea-denaturing polyacrylamide gel electrophoresis (PAGE) and detected by a Pharos FX Plus Imager (Bio-rad Laboratories, Hercules, CA).

2.4. Measurement of APE1 activity in removing an abasic site adjacent to a T/G mismatch

APE1 incision of the THF residue, an abasic site analog, that is adjacent to 5mC or a T/G mismatch, was measured at 37 °C in a 20 μ l reaction mixture containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 0.1 mg/ml BSA, 5 mM MgCl₂, and 0.01% Nonidet

P-40. APE1 activity was examined by incubating 5 nM APE1 with 25 nM substrate containing the THF residue adjacent to a mismatched T or matched C/G in the absence and presence of increasing concentrations of TDG (10, 25, 50 and 70 nM). Reactions were terminated by transferring to 95 °C for 5 min in stopping buffer containing 95% formamide and 10 mM EDTA. Substrates and products were separated by 15% urea-denaturing PAGE and detected by a Pharos FX Plus Imager.

2.5. Measurement of pol β DNA synthesis activity in extending the 3'-terminus mismatched T of a T/G mismatch

Pol β DNA synthesis on a single-nucleotide gapped substrate to extend a 3'-mismatched T or 3'-matched C was measured using a one-nucleotide gapped substrate containing a T/G mismatch or a C/G match at the 3'-terminus of the upstream primer and a 5'-THF residue at the downstream primer. Enzymatic reactions were performed by incubating 25 nM substrate with various concentrations of pol β at 37 °C for 15 min in a 10 μ l reaction mixture containing BER buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 0.1 mg/ml BSA, 0.01% Nonidet P-40, 5 mM MgCl₂). APE1 3'-5' exonuclease activity of removing the T from the 3'-T/G mismatch was measured in BER buffer at 37 °C for 15 min. FEN1 cleavage activity on the THF residue was examined in BER reaction buffer with 50 μ M dNTPs in the absence or presence of 5 nM pol β at 37 °C for 15 min. Reactions were terminated by transferring to 95 °C for 5 min in stopping buffer containing 95% formamide and 10 mM EDTA. Substrates and products were separated by 15% urea-denaturing PAGE and detected by a Pharos FX Plus Imager.

2.6. Measurement of the efficiency of pol β and APE1 activities in the context of a 3'-T/G mismatch

The efficiency of pol β and APE1 in extending or removing a 3'-terminus mismatched T was determined by measuring pol β gap-filling synthesis activity and APE1 3'-5' exonuclease activity at various time intervals. Reactions were conducted by incubating 25 nM 5'-³²P-labeled substrate with 5 nM pol β or increasing concentrations of APE1 alone at 5 nM, 50 nM, and 100 nM. The reaction mixture was assembled on ice and incubated at 37 °C for 1, 2, 5, 10, 15, and 30 min intervals. Reactions were subsequently terminated by transferring to 95 °C for 5 min in stopping buffer containing 95% formamide and 10 mM EDTA. Substrates and products were separated by 15% urea-denaturing PAGE and detected by a Pharos FX Plus Imager.

2.7. *In vitro* reconstituted BER assay

BER in the context of a T/G mismatch was reconstituted with purified APE1, pol β , FEN1, LIG I and the 1 nt-gapped substrate with a 3'-terminus matched C/G or T/G mismatch on the upstream primer, and a THF residue on the downstream primer opposite a template C or T. Ten microliter reaction mixture contained BER buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 0.1 mg/ml BSA, 0.01% Nonidet P-40, 5 mM MgCl₂), 25 nM substrate, 5 mM Mg²⁺, 50 μ M dNTPs, 2 mM ATP and the indicated amounts of BER enzymes. The reaction mixture was assembled on ice and incubated at 37 °C for 15 min. Reactions were terminated by transferring to 95 °C for 5 min in stopping buffer containing 95% formamide

and 10 mM EDTA. Substrates and products were separated by 15% urea-denaturing PAGE and detected by a Pharos FX Plus Imager.

2.8. Sequencing of repair products resulting from BER of an abasic lesion adjacent to a T/G mismatch

BER of an abasic lesion adjacent to a T/G mismatch was performed by incubating 50 nM purified APE1, 5 nM pol β , 50 nM FEN1, and 5 nM LIG I with 25 nM 1 nt-gapped substrate with a 3'-terminus T/G mismatch on the upstream primer, and a 5'-THF residue on the downstream primer. The BER reaction (20 μ l) was reconstituted with the indicated concentrations of BER enzymes and substrates in BER reaction buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 0.1 mg/ml BSA, 0.01% Nonidet P-40, 5 mM MgCl₂) that contained 50 μ M dNTPs, 5 mM Mg²⁺ and 2 mM ATP. The reaction mixtures were assembled on ice, and incubated at 37 °C for 15 min. Reactions were then terminated by transferring to 95 °C for 10 min. To isolate the repair products, the template strand of the substrate was biotinylated at the 5'-end. The repair products were incubated with avidin agarose beads (Pierce-Thermo Scientific, Rockford, IL) in binding buffer that contained 0.1 M phosphate, 0.15 M NaCl, pH 7.2 and 1% Nonidet P-40 at 4 °C for 2 h with rotation. The agarose beads were centrifuged at 5000 rpm for 1 min and were washed three times with binding buffer. The repaired strands were then separated from their template strands through incubation in 0.15 M NaOH for 15 min with rotation under room temperature, followed by centrifugation at 5000 rpm for 2 min. The repaired strands were then precipitated with ethanol and dissolved in TE buffer. The repair products were then subjected to PCR amplification with a forward primer (5'-GCA GTC CTC TAG TCG TAG TAG-3') and a reverse primer (5'-GCA ATG AGT AAG TCT AGC TAC TAC-3'). PCR amplification was performed under the following conditions: 95 °C for 5 min, 1 cycle; 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, 35 cycles; 72 °C for 30 min. The PCR products were then subjected to TA cloning using a TA cloning kit by following the manufacturer's instructions (Thermo Fisher Scientific, Pittsburgh, PA). White colonies were picked for amplifying plasmids that contained the inserts (repair products). Plasmids were isolated with a Miniprep plasmid isolation kit (Promega, Madison, WI), dissolved in TE buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA), and subjected to DNA sequencing. Sequencing reactions were performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Pittsburgh, PA) and were subjected to capillary electrophoresis (Florida International University DNA Sequencing Core Facility). Sequencing results were analyzed by MacVector 12.5.1 (MacVector, Apex, NC).

3. Results

3.1. TDG is completely inhibited by an abasic lesion

In CpG dinucleotide clusters, a 5mC can be converted to a T during active DNA demethylation, thereby resulting in a T/G mismatch [27]. On the other hand, guanines serve as hot spots of oxidative DNA damage, and guanines located in CpG dinucleotide clusters can be damaged leading to the production of a base lesion such as an abasic site adjacent to the 5mC. However, it remains unknown whether an abasic lesion can affect TDG removal of a T/G mismatch. To test this possibility, we initially examined the removal of the

mismatched T from a T/G mismatch adjacent to an abasic site by TDG. A substrate containing a mismatched T/G adjacent to an abasic site analog, a THF residue, was used to mimic the intermediate with an abasic lesion generated after deamination of 5mC. This would allow TDG to remove the 3'-mismatched T generating a native abasic site, which was subsequently broken by a high temperature at 95 °C into single-strand break intermediates as TDG products. The results showed that 50 nM TDG removed the mismatched T next to a normal G (Fig. 1, lane 2). Its activity was significantly increased with increasing concentrations of TDG from 60 nM to 70 nM (Fig. 1, lanes 3–4). Surprisingly, the same concentrations of TDG (50–70 nM) failed to remove a mismatched T next to an abasic site (Fig. 1, lanes 6–8). This indicated that TDG activity was completely inhibited by an abasic lesion adjacent to a T/G mismatch. This further suggests that the presence of an abasic lesion next to an intermediate of 5mC demethylation in CpG dinucleotide clusters can lead to accumulation of T/G mismatches.

3.2. APE1 can efficiently incise an abasic site that is adjacent to a T/G mismatch in the presence of TDG

To further determine if a 3'-mismatched T affects APE1 activity, APE1 5'-incision of an abasic site adjacent to a T/G mismatch was examined in the absence and presence of TDG. Substrates containing a 3'-mismatched T next to a THF residue opposite to a C or a T were used for measuring APE1 activity. The substrates mimic the intermediates generated by deamination of the 5mC on the damaged strand alone or the 5mCs on both the damaged strand and template strand simultaneously. The results showed that 5 nM APE1 efficiently carried out 5'-incision of an abasic site in the absence and presence of TDG (Fig. 2, lanes 1–6 and lanes 7–12).

3.3. Pol β can efficiently extend the 3'-mismatched T of a T/G mismatched base pair

Since APE1 5'-incision of an abasic site next to a T/G mismatch resulted in the 1-nt gap intermediate with a 3'-mismatched T, which would be subsequently passed to pol β for continuation of repair of the lesion, we then asked if pol β could still perform DNA synthesis in the presence of a 3'-mismatched T. To test this, we examined pol β gap-filling synthesis using the substrates with a 3'-matched C or a 3'-mismatched T adjacent to a pre-incised THF residue or a 3'-T along with a template T opposite a preincised THF residue. We found that 1 nM, 5 nM and 10 nM pol β efficiently extended the 3'-matched C (Fig. 3, lanes 2–4). However, surprisingly, pol β also efficiently extended a 3'-mismatched T (Fig. 3, lanes 6–8 and lanes 10–12), particularly at the concentrations of 5 nM and 10 nM (Fig. 3, lanes 7–8 and 11–12). For all substrates, pol β mainly inserted one nucleotide to extend the 3'-terminus T and fill in the gap. Interestingly, pol β extension of a 3'-mismatched T with a template T was much weaker than its synthesis with a template C, indicating that the polymerase had difficulty in extending a 3'-mismatched T when it incorporated A to base pair with a template T. The results further suggest that pol β can tolerate a 3'-terminus mismatched nucleotide, similar to DNA translesion synthesis polymerases such as pol κ and pol μ that exhibit mismatch tolerance [37,38]. Moreover, pol β extension of a 3'-terminus matched C, but not 3'-mismatched T resulted in small amount of products with more than one nucleotide insertion (Fig. 3, lanes 3–4). This indicates that pol β performed strand-displacement synthesis to extend a 3'-matched C, however it failed to perform this synthesis when it

extended a 3'-mismatched T indicating that the mismatched base inhibited further pol β DNA synthesis. In conclusion, pol β tolerated and efficiently extended a 3'-mismatched T resulting from deamination of a 5mC in CpG dinucleotides during BER.

3.4. APE1 3'-5' exonuclease activity efficiently removes a 3'-mismatched T

It has been reported that APE1 3'-5' exonuclease activity can remove mismatched nucleotides [39–41]. Thus, we further hypothesized that APE1 might also remove the 3'-mismatched T from a T/G mismatch to increase the fidelity of pol β in a CpG dinucleotide. To test this possibility, we examined APE1 3'-5' exonuclease activity on the substrate (25 nM) with a 3'-matched C or 3'-mismatched T on the upstream strand and a 5'-THF residue on the downstream strand with a template C or T opposite an abasic site. We found that APE1 (5 nM, 10 nM, 25 nM and 50 nM) exhibited poor cleavage activity to remove the 3'-matched C (Fig. 4, lanes 2–5 and the quantification of the APE1 3'-5' exonuclease cleavage products shown below the gel), indicating inefficient removal of a 3'-matched nucleotide by APE1 3'-5' exonuclease activity. However, APE1 removed the 3'-mismatched T much more efficiently than it removed a 3'-matched C (Fig. 4, lanes 7–10 and 12–15 and the quantification of the APE1 3'-5' exonuclease cleavage products shown below the gel). For all the substrates with a T/G mismatch, APE1 mainly removed one nucleotide from the upstream primer containing a 3'-mismatched T. Thus, APE1 3'-5' exonuclease efficiently removed the 3'-mismatched T. Interestingly, we found that APE1 3'-5' exonuclease exhibited much weaker activity in removing the 3'-mismatched T located next to a template T than in removing the mismatched nucleotide next to a C on the template strand (Fig. 4, lanes 12–15), indicating that simultaneous demethylation of 5mCs on both the damaged and template strands also decreased APE1 3'-5' exonuclease activity.

3.5. FEN1 cleaves a sugar phosphate flap adjacent to a 3'-T/G mismatch

To further determine whether a 3'-terminus mismatched T will have any effect on FEN1 cleavage, we characterized FEN1 cleavage activity with the substrates (25 nM) containing an upstream 3'-C or 3'-T and a downstream 5'-THF residue and a template C or T in the absence and presence of pol β . We found that in the absence of pol β , FEN1 (5 nM, 10 nM and 25 nM) exhibited poor cleavage activity. It mainly cleaved a flap containing one nucleotide on all the substrates (Fig. 5, lanes 2–4, lanes 9–11, lanes 16–18). However, a high concentration of FEN1 (25 nM) managed to cleave a 5 nt-flap (Fig. 5, lane 4, 11, 18). This suggests that the dynamic DNA in the context of CG dinucleotides on the template strand facilitated the formation of a flap on the downstream strand. In the presence of pol β DNA synthesis, FEN1 flap cleavage was significantly stimulated with the substrates containing a 3'-matched C and 3'-mismatched T. The enzyme cleaved flaps containing 12 to 14 nucleotides (Fig. 5, lanes 5–7 and lanes 12–14). This indicated that pol β strand-displacement synthesis created a longer flap for FEN1 cleavage. However, the presence of pol β DNA synthesis did not significantly improve FEN1 flap cleavage on the substrate with a template T (Fig. 5, lanes 19–21). This indicated that FEN1 exhibited weak cleavage of a flap next to a template in the presence of pol β . This appeared to result from the inhibition of pol β strand-displacement synthesis by a 3'-T/G mismatch (Fig. 3).

3.6. The efficiency of pol β extension of a 3'-mismatched T and APE1 3'-5' exonuclease to remove the 3'-mismatched T

Since our previous results indicate that removal of a 3'-mismatched T by APE1 is critical for improving pol β fidelity, we then asked if APE1 could remove a 3'-mismatch T before pol β could extend the mismatched nucleotide during BER. We tested this by examining the efficiency of pol β DNA synthesis at 5 nM and that of APE1 3'-5' exonuclease activity at 5 nM, 50 nM and 100 nM using the substrate containing a 3'-mismatched T and a 5'-THF residue with a template C opposite the THF residue. The enzymatic reaction products were measured at different time intervals ranging from 0 min to 30 min. The percentage of products was plotted against time (Fig. 6). The results revealed that 5 nM pol β extended a 3'-mismatched T much more efficiently than APE1 3'-5' exonuclease removed the mismatched nucleotide at the same concentration. However, the 3'-5' exonuclease activity of APE1 at 50 nM and 100 nM removed the mismatched T much more efficiently than 5 nM pol β extended the mismatched nucleotide (Fig. 6). This indicated that a high concentration of APE1 was sufficient to combat pol β extension of a mismatched nucleotide. Since APE1 is much more abundant than pol β in mammalian cells, the results further suggest that APE1 can remove a mismatched T before it is extended by pol β in cells.

3.7. BER can occur in the presence of 3'-T/G mismatch

To examine whether a 3' T/G mismatch can ultimately result in the production of BER products, we reconstituted BER by incubating the substrates without or with a T/G mismatch with APE1, pol β , FEN1 and LIG I. We found that a significant amount of repair products were produced during BER with a 3'-mismatched T in the absence of APE1 (Fig. 7A, lanes 6 and 10) although the amount of products was less than those formed from the substrate with a 3'-matched C (Fig. 7A, lane 2). This indicated that in the absence of APE1, pol β extended a 3'-mismatched T resulting in repair product with a T/G mismatch. We found that presence of high concentrations of APE1 (50 nM and 100 nM) significantly stimulated the production of repair products (Fig. 7A, lanes 7–8) during BER with the substrate containing a 3'-mismatched T and a template C. However, this effect was not evident with the substrate containing a 3'-mismatched T and a template T (Fig. 7A, lanes 11–12). This indicated that BER was accomplished through pol β tolerance of a 3'-mismatched T suggesting that this can further result in a C to T mutation, and APE1 may reduce the production of the mutation.

3.8. APE1 significantly reduces C to T mutations resulting from a T/G mismatch during BER

To further confirm whether APE1 can reduce mutations resulting from extension of a 3'-mismatched T by pol β during BER, we sequenced the repair products generated from reconstituted BER with the substrates containing a 3'-mismatched T adjacent to a THF residue opposite a template C or T in the absence and presence of APE1. The results showed that in the absence of APE1, all repair products (100%) contained a mismatched T (Fig. 7B). This indicated that the mismatched T was fully extended by pol β during BER. However, in the presence of 50 nM APE1, only 7.7%–13% of the repair products contained a mismatched T, and 87%–92.3% of the mismatched Ts were converted into matched Cs (Fig.

7B). This indicated that APE1 3'-5' exonuclease activity efficiently removed mismatched Ts allowing pol β to insert a C to base pair with a template G during BER. Taken together, our results indicated that APE1 3'-5' exonuclease significantly reduced T/G mismatches, thereby preventing mutations in CpG dinucleotides during BER.

4. Discussion

In this study, for the first time, we explored the role of BER in maintaining the integrity of CpG islands by removing a mismatched base when DNA base damage occurs next to a T/G mismatch resulting from deamination of a 5mC in a CpG dinucleotide. Since CpGs are susceptible to oxidative DNA damage, a DNA base lesion such as 8-oxoG or an abasic site can be readily generated next to a T/G mismatch. In this scenario, both the mismatched T and base lesion need to be resolved by the BER pathway. Thus an important issue is how BER, in the context of the two different types of base lesions, may be initiated. It has been shown that OGG1 removes an 8-oxoG next to a 5'-T/G mismatch about 3.5-fold faster than TDG removes the mismatched T [36]. This indicates that the 8-oxoG is usually removed by OGG1 prior to the removal of the mismatched T by TDG. This results in an abasic site next to the 5'-T/G mismatch. Surprisingly, in this study, we found that the activity of TDG of removing the mismatched T adjacent to an abasic site was completely inhibited by the abasic lesion (Fig. 1, lanes 6–8) indicating that an abasic lesion next to the mismatched nucleotide completely suppressed the removal of the mismatched T by TDG. However, we showed that APE1 incised an abasic lesion efficiently in the presence of the mismatched T (Fig. 2, lanes 3–6 and lanes 9–12) leading to the production of a single-strand break intermediate with the 3'-terminus mismatched T. We found that pol β efficiently extended the 3'-mismatched T (Fig. 3, lanes 6–8 and lanes 10–12), thereby resulting in maintenance of the mismatched base in the repair products (Fig. 7A, lanes 6–8 and lanes 10–12). This subsequently led to a C to T transition mutation in the repair products (Fig. 7B). However, the pol β -mediated mutagenic effect was significantly reduced (Fig. 7B) through the removal of the 3'-mismatched T by APE1 3'-5' exonuclease activity (Fig. 4, lanes 7–10 and lanes 12–15 and Fig. 6). Our results support a model whereby a base lesion such as an abasic lesion occurs adjacent to a T/G mismatched base pair in a CpG dinucleotide during DNA demethylation. Removal of the mismatched T by TDG is completely inhibited by the abasic lesion. This allows APE1 to incise the abasic site at its 5'-end to initiate BER. This subsequently leads to a 1nt gapped DNA with a 3'-mismatched T that can be subjected to two different subpathways (Fig. 8). If the 3'-mismatched T is captured by pol β before it is removed by APE1 3'-5' exonuclease activity, it is extended by pol β . This then results in the sustainment of the 3'-mismatched T during BER, thereby causing a C to T transition mutation (Fig. 8, subpathway 1). However, if the 3'-mismatched T is removed by APE1 3'-5' exonuclease activity before it is extended by pol β , pol β fills in the gap by incorporating a correct nucleotide to accomplish BER (Fig. 8, subpathway 2). In this scenario, APE1 3'-5' exonuclease activity prevents mutation during BER in the context of a T/G mismatch DNA demethylation intermediate.

Here, we provided the first evidence that APE1 3'-5' exonuclease can prevent C to T mutations during BER of an abasic lesion adjacent to a T/G mismatch in a CpG dinucleotide, thereby maintaining the integrity of CpGs. APE1 3'-5' exonuclease activity

was initially reported to be able to remove a 3'-mismatched nucleotide in DNA [39]. It has been found that the efficiency of the 3'-5' exonuclease in removing a mismatched nucleotide is 160-fold higher than that in removing a matched nucleotide [39]. Thus, it has been proposed that APE1 3'-5' exonuclease serves as a proofreading enzyme for correcting mismatched nucleotides incorporated by pol β . This is further suggested by a previous finding showing that APE1 forms a complex with pol β on a nicked or 1 nt gapped DNA with a deoxyribonucleotide phosphate (dRP) flap [41]. Although the APE1-pol β -DNA ternary complex failed to significantly stimulate APE1 3'-5' exonuclease activity on the nicked DNA [41], it is possible that the complex may facilitate APE1 3'-5' exonuclease activity at a gapped DNA with a sugar phosphate. Moreover, our results showed that high concentrations of APE1 removed the 3'-mismatched T more efficiently than pol β extension of the 3'-mismatched nucleotide. Thus, it is conceivable that a relatively high concentration of APE1 in mammalian cells exhibits a robust 3'-5' exonuclease activity that can cooperate with pol β to remove a T/G mismatch in CpGs and ensure that the polymerase inserts a correct nucleotide. Thus, sufficient amounts of APE1 in mammalian cells should lead to prevention of C to T transition mutations resulting from DNA demethylation during BER, thereby sustaining epigenome stability.

Our results also showed that APE1 cleavage activity on an abasic site was not affected by the presence of increasing concentrations of TDG (Fig. 2). This further suggests that APE1 predominantly bound to the abasic site next to the mismatched T, kicking TDG off the mismatched nucleotide during BER. Our results further demonstrated that APE1 5'-incision of an abasic site opposite a template T generated the same size of the product as the one from its cleavage on the abasic site opposite a template C. This suggests that TDG activity was inhibited by an adjacent abasic site independent of the template nucleotide. Moreover, we showed that APE1 exhibited a lower 3'-5' exonuclease activity with the substrate containing a template T that is opposite the abasic site (Fig. 4, lanes 12–15). This suggests that the template T modulated the structure of the substrate, and this affected APE1 binding to the 3'-mismatched T, thereby reducing its 3'-5' exonuclease activity. However, our DNA sequencing results showed that 50 nM APE1 effectively removed 87% of 3'-mismatched T with the substrate containing a template T, thereby preventing T/G mutation. This demonstrated that 10-fold of APE1 that of pol β was sufficient for removing the 3'-mismatched T in the context of a template T. In the scenario where pol β is over-expressed in tumor cells, the ratio between APE1 and pol β may be reduced. Therefore it is conceivable that more T/G mismatch mutations could be generated by pol β in the tumor cells.

Our results showed that pol β efficiently extended a mismatched T basepaired with a template G that is adjacent to a single-nucleotide gap. This is consistent with the findings from a study from the Wilson group [42]. A study from the Sweasy group has shown that pol β exhibits low fidelity in gap-filling and strand displacement synthesis [43]. Pol β can extend a T basepaired with a G adjacent to a 6 nt gap, albeit with a low efficiency compared with extension of a matched C [43]. All these findings indicate that pol β can tolerate a 3'-terminus mismatch next to a single-nucleotide gap. A translesion synthesis polymerase, pol κ , has been found to extend a 3'-mismatched T via a primer-template misalignment or direct extension [37]. However, our results showed that pol β could extend a mismatched T only by

direct extension, suggesting that pol β interacted with the 3'-mismatched T and the template strand in a more rigid manner than pol κ .

Our study has also provided new insight into a mechanism underlying mutations in CGIs that can be readily generated through BER and BER-mediated DNA demethylation. We have demonstrated that in the scenario where 3'-mismatched T is adjacent to an abasic site in a CpG dinucleotide, the mismatched T was efficiently removed by APE1 before pol β extended it (Fig. 7B), and a C to T mutation was prevented (Fig. 7B). However, in the scenario where a T occurs at the template strand and is opposite an abasic site (Figs. 2–5 and Fig. 7), the T could not be removed by APE1 3'–5' exonuclease. Instead, pol β filled in the gap by inserting an A to basepair with the template T leading to a C to T transition. Thus, in this scenario, C to T mutations cannot be prevented by APE1 3'–5' exonuclease. Our discoveries provide new mechanistic evidence underlying the high frequency of mutations at CGIs. In conclusion, our study demonstrates that APE1 exonuclease activity functions as a proofreader for pol β to sustain the integrity of CGIs during BER and DNA demethylation by removing a 3'-terminus T/G mismatch.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.dnarep.2016.03.006>.

Fig. 1.

The activity of TDG in removing a T/G mismatch at a CpG dinucleotide is inhibited by an adjacent abasic lesion. An oligonucleotide substrate that contained random DNA sequence with a THF residue next to a 5'-T/G mismatch in a CpG dinucleotide was used to mimic a BER intermediate generated from removal of 8-oxoG by OGG1 and deamination of 5mC. The substrate that contained a G with a 5'-T/G mismatch was employed to mimic an intermediate generated by deamination of 5mC alone. TDG activity in removing a T/G mismatch was measured as described in "Materials and methods". Substrates were ³²P-labeled at the 5'-end of the damaged strand. Lanes 1 and 5 correspond to the substrate only. Lanes 2 and 6 correspond to reaction mixtures with 50 nM TDG. Lanes 3 and 7 correspond to reaction mixtures with 60 nM TDG. Lanes 4 and 8 correspond to reaction mixtures with 70 nM TDG. TDG cleavage products are indicated. Substrates are illustrated schematically above the gel.

Fig. 2.

APE1 can efficiently incise an abasic site that is adjacent to a T/G mismatch in a CpG dinucleotide in the presence of TDG. APE1 5'-incision of an abasic site that is adjacent to a 5'-T/G mismatch in a CpG dinucleotide was examined under the conditions described in "Materials and methods". Substrates, as described in Fig. 1, were ³²P-labeled at the 5'-end of the damaged strand. Lanes 1 and 7 correspond to the substrate only. Lanes 2 and 8 correspond to reaction mixtures with 5 nM APE1 alone. Lanes 3 and 9 correspond to reaction mixtures with 5 nM APE1 in the presence of 10 nM TDG. Lanes 4 and 10 correspond to reaction mixtures with 5 nM APE1 in the presence of 25 nM TDG. Lanes 5 and 11 correspond to reaction mixtures with 5 nM APE1 in the presence of 50 nM TDG. Lanes 6 and 12 correspond to reaction mixtures with 5 nM APE1 in the presence of 75 nM TDG. Substrates and APE1 cleavage products are indicated. The substrates are illustrated schematically above the gel.

Fig. 3.

Pol β extends a 3'-terminus mismatched T at a CpG dinucleotide. Pol β DNA synthesis in the context of a T/G mismatch was examined as described in "Materials and methods".

Substrates were designed to mimic a BER intermediate containing a 3'-terminus matched C, or 3'-terminus mismatched T in a CpG dinucleotide with an abasic site preincised by APE1 opposite to a C or T on the template strand. Substrates were ^{32}P -labeled at the 5'-end of the damaged strand. Lanes 1, 5, and 9 correspond to the substrate only. Lanes 2, 6, and 10 correspond to reaction mixtures with 1 nM pol β . Lanes 3, 7, and 11 correspond to reaction mixtures with 5 nM pol β . Lanes 4, 8, and 12 represent reaction mixtures with 10 nM pol β . Substrates and pol β DNA synthesis products are indicated. Substrates are illustrated schematically above the gel.

Fig. 4.

APE1 3'-5' exonuclease activity removes a 3'-terminus mismatched T in a CpG dinucleotide. APE1 3'-5' exonuclease activity in removing a 3'-mismatched T in a CpG dinucleotide was measured as described in "Materials and methods". Substrates, as described in Fig. 3, were ^{32}P -labeled at the 5'-end of the damaged strand. Lanes 1, 6 and 11 correspond to the substrate only. Lanes 2, 7 and 12 correspond to reaction mixtures with 5 nM APE1. Lanes 3, 8 and 13 correspond to reaction mixtures with 10 nM APE1. Lanes 4, 9 and 14 correspond to reaction mixtures with 25 nM APE1. Lanes 5, 10 and 15 correspond to reaction mixtures with 50 nM APE1. Substrates and APE1 cleavage products are indicated. Substrates are illustrated schematically above the gel. The quantification of the APE1 3'-5' exonuclease cleavage products are shown below the gels.

Fig. 5.

FEN1 flap cleavage in the presence of a T/G mismatch in a CpG dinucleotide. FEN1 flap cleavage activity in the presence of a T/G mismatch in a CpG dinucleotide was examined as described in “Materials and methods”. Substrates, as described in Fig. 3, were ^{32}P -labeled at the 3'-end of the damaged strand. Lanes 1, 8 and 15 correspond to the substrate only. Lanes 2, 9 and 16 correspond to reaction mixtures with 5 nM FEN1. Lanes 3, 10 and 17 correspond to reaction mixtures with 10 nM FEN1. Lanes 4, 11 and 18 correspond to reaction mixtures with 25 nM FEN1. Lanes 5, 12 and 19 correspond to reaction mixtures that contained 5 nM FEN1 in the presence of 5 nM pol β . Lanes 6, 13 and 20 represent reaction mixtures that contained 10 nM FEN1 in the presence of 5 nM pol β . Lanes 7, 14 and 21 represent reaction mixtures that contained 25 nM FEN1 in the presence of 5 nM pol β . Substrates and FEN1 cleavage products are indicated. Substrates are illustrated schematically above the gel.

Fig. 6.

The activities of pol β DNA synthesis and APE1 3'–5' exonuclease in the context of a T/G mismatch in a CpG dinucleotide. The activities of pol β and APE1 in extending or removing a 3'-terminus mismatched T were determined at various time intervals (1, 2, 5, 10, 15 and 30 min) with a substrate containing a 3'-terminus mismatched T in a CpG dinucleotide with a THF residue preincised by APE1 as described in "Materials and methods". The amount of products resulting from pol β DNA synthesis or APE1 3'–5' exonuclease activity were quantified by using Quantity One Software (Bio-Rad Laboratories, Hercules, CA). The percentage of products was calculated as the amount of products over total amount of substrates and was plotted against time.

Fig. 7. BER reconstitution in the context of a T/G mismatch in a CpG dinucleotide. (A) BER reconstitution in the context of a T/G mismatch was performed as described in “Materials and Methods”. Substrates, as described in Fig. 3, were ^{32}P -labeled at the 5'-end of the damaged strand. Lanes 1, 5 and 9 correspond to substrate only. Lanes 2, 6 and 10 correspond to reaction mixtures that contained 5 nM pol β , 50 nM FEN1 and 5 nM LIG I. Lanes 3, 7 and 11 correspond to reaction mixtures that contained 50 nM APE1, 5 nM pol β , 50 nM FEN1 and 5 nM LIG I. Lanes 4, 8 and 12 correspond to reaction mixtures that contained 100 nM APE1, 5 nM pol β , 50 nM FEN1 and 5 nM LIG I. Substrates and repair products are indicated. Substrates are illustrated schematically above the gel. (B) DNA sequencing results of repair products resulting from BER of a T/G mismatch and abasic lesion in a CpG

dinucleotide. Repair products were separated from the template strand with avidin beads. Subsequently, repair products were amplified by PCR and cloned into a TA vector and subjected to DNA sequencing. The percentage of repair products that contained a mismatched T or matched C from the different substrates are shown.

Fig. 8.

APE1 3'-5' exonuclease combats the extension of a T/G mismatch by pol β and C to T mutations in CpGs during BER. During active DNA demethylation of 5mC in CpG dinucleotides, the 5mC is deaminated to T thereby leading to production of a T/G mismatch, while the adjacent G can also be damaged by oxidative stress resulting in an abasic lesion. TDG activity in removing the T/G mismatch is completely inhibited by the abasic lesion. This allows the accumulation of T/G mismatches that are adjacent to abasic lesions. APE1 can efficiently incise the resulting abasic site, leading to a 1nt gap with a 3'-terminus mismatched T. Pol β can extend the mismatched T to create a short flap with a sugar phosphate. Subsequently, FEN1 removes the flap, generating a nick for ligation by DNA ligase I. This leads to a C to T transition mutation (Sub-pathway 1). In a scenario where the 3'-terminus mismatched T is efficiently removed by APE1 3'-5' exonuclease prior to pol β extension of the mismatch nucleotide, pol β incorporates a C to basepair with a template G. This prevents a C to T mutation (Sub-pathway 2).