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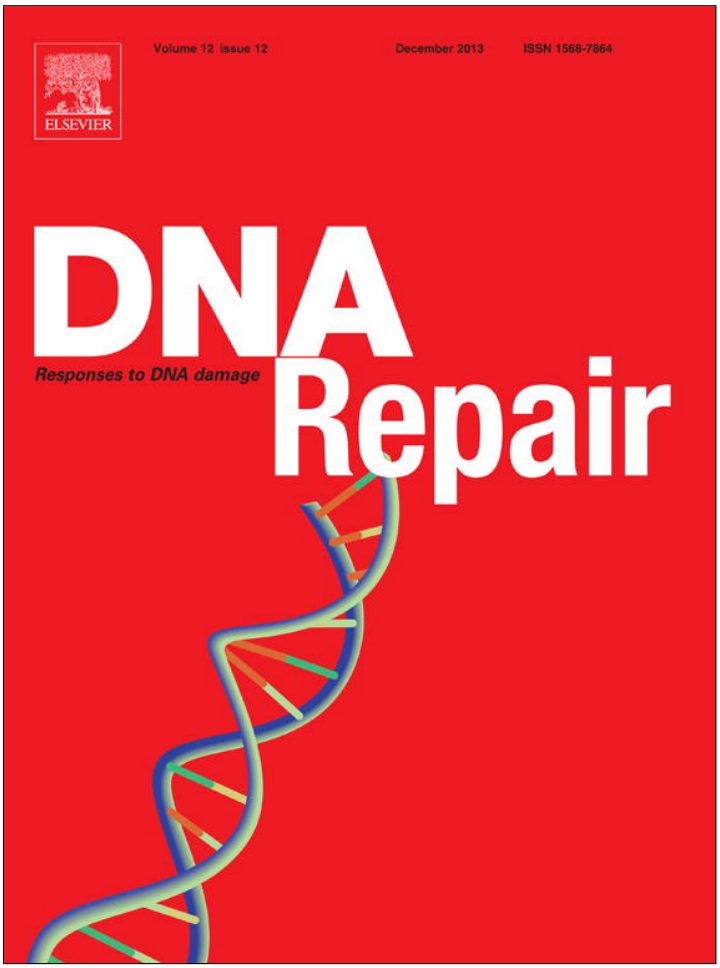

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The mechanism of human tyrosyl-DNA phosphodiesterase 1 in the cleavage of AP site and its synthetic analogs

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ABSTRACT

The mechanism of hydrolysis of the apurinic/apyrimidinic (AP) site and its synthetic analogs by using tyrosyl-DNA phosphodiesterase 1 (Tdp1) was analyzed. Tdp1 catalyzes the cleavage of AP site and the synthetic analog of the AP site, 3-hydroxy-2(hydroxymethyl)-tetrahydrofuran (THF), in DNA by hydrolysis of the phosphodiester bond between the substituent and 5′ adjacent phosphate. The product of Tdp1 cleavage in the case of the AP site is unstable and is hydrolyzed with the formation of 3′- and 5′-margin phosphates. The following repair demands the ordered action of polynucleotide kinase phosphorylase, with XRCC1, DNA polymerase β, and DNA ligase. In the case of THF, Tdp1 generates break with the 5′-THF and the 3′-phosphate termini. Tdp1 is also able to effectively cleave non-nucleotide insertions in DNA, decanediol and diethyleneglycol moieties by the same mechanism as in the case of THF cleavage. The efficiency of Tdp1 catalyzed hydrolysis of AP-site analog correlates with the DNA helix distortion induced by the substituent. The following repair of 5′-THF and other AP-site analogs can be processed by the long-patch base excision repair pathway.

1. Introduction

Tyrosyl-DNA phosphodiesterase 1 (Tdp1) is a member of the phospholipase D family that removes DNA-adducts within single-strand breaks (SSB) and double-strand breaks (DSB). The unique enzymatic activity of Tdp1 removes the covalently linked adducts of DNA topoisomerase I (Top1) from the DNA 3′-end. It is supposed that Top1 covalently linked to DNA has to be denatured or proteolysed before the action of Tdp1 [1,2]. Tdp1 activity is not limited to the removal of cellular Top1 adducts; it can also process other 3′ DNA end-blocking lesions: 3′ abasic site (3′ tetrahydrofuran moiety) or bulky substituents and can use 3′ phosphoglycolate as substrate [3–5]. Tdp1 is now regarded as a general 3′ DNA end-processing enzyme that acts within the SSB repair complex to remove adducts and to prepare the broken DNA ends bearing a 3′ phosphate group for further processing by repair enzymes [6]. Tdp1 also possesses a limited DNA and RNA 3′-nucleosidase activity by which a single nucleoside is removed from the 3′-hydroxyl end of the substrate [4,7]. Thus, Tdp1 may function to remove a variety of adducts from 3′-DNA ends during DNA repair [4,7–9].

Recently we have shown that human Tdp1 can cleave an apurinic/apyrimidinic (AP) site located inside a DNA strand with the formation of 3′-phosphate terminus, which suggests a novel APE-independent pathway for the processing of AP sites [10,11]. Abasic sites are one of the most frequent endogenous lesions in cellular DNA and their repair is critical to provide genome stability and cell survival. The major enzymes involved in incision of AP sites are AP endonucleases initiating base excision repair (BER) by hydrolyzing the DNA on the 5′ side of the AP site leaving a single-stranded (ss) nick with a 3′-hydroxyl (3′-OH) and a 5′-deoxyribose phosphate (5′-dRP) ends [12]. Bifunctional AP lyases/DNA glycosylases may also initiate repair of AP sites. AP lyases cleave the DNA strand on the 3′ side of the AP site by β-elimination generating a 5′-P and a 3′-phospho-α,β-unsaturated aldehyde (3′-PUA) termini [13]. The 3′-PUA residue is further processed by an AP endonuclease leaving a 3′-OH terminus. In the recent study by Nilsen and co-workers, it was demonstrated that Schizosaccharomyces pombe Tdp1 is able to remove 3′-α,β-unsaturated aldehyde [14]. We also showed that this residue can be removed by the activity of human Tdp1 [10]. Further, the blocking 3′-phosphate is removed by the phosphatase activity of the polynucleotide kinase 3′-phosphatase (PNKP) to produce a 3′-hydroxyl, which can be processed by DNA polymerases

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and ligases [15–17]. Therefore, the AP site can be repaired independently of AP endonuclease activity. Tdp1 was detected in the complex with several BER enzymes, notably Pol β, XRCC1, PARP1, PNKP, and DNA ligase III [8,18,19]. The Tdp1-induced BER pathway may be regarded as one of the back-up repair mechanisms providing genomic DNA stability.

To further understand how Tdp1 cleaves a DNA structure containing an AP site, we have examined the ability of Tdp1 to hydrolyze DNA containing synthetic analogs of the AP site – tetrahydrofuran (THF) and non-nucleotide insertions into DNA, decanediol (DD) and diethyleneglycol (DEG) moieties in comparison with natural AP sites. In the case of natural AP sites we detected phosphates at the 3′ and 5′ margins of resulting break [10]. However in the case of synthetic analogs of the AP site the 3′-margin phosphate and 5′-residues (THF, DD, and DEG) were generated. The long patch (strand displacement) DNA repair synthesis that occurs after cleavage of synthetic AP sites by Tdp1 was analyzed.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (5000 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were produced in the Laboratory of Radiochemistry (ICBFM, Novosibirsk); [α-^{32}P]-3′-deoxyadenosine 5′-triphosphate, (cordycepin 5′-triphosphate) (250 μCi) was purchased from PerkinElmer Life Sciences (USA). Terminal deoxynucleotidyl transferase (TdT) was purchased from Fermentas (Lithuania); phage T4 polynucleotide kinase was purchased from Biosan (Russia); reagents for electrophoresis and buffer components from Sigma (USA). Ultrapure dNTPs were from SibEnzyme (Russia).

The recombinant wild-type Tdp1 was purified to homogeneity by the chromatography on Ni-chelating resin and phosphocellulose P11 as described [11,20]. The recombinant purified UDG, APE1, FEN1, and DNA polymerase β (Polβ) were a generous gift from Dr. S.N. Khodyreva (ICBFM, Novosibirsk). Histidine-tagged human PNKP was purified to homogeneity on Ni-chelating resin as described by the manufacturer followed by chromatography on sulfopropyl-sepharose. The recombinant plasmid coding PNKP was a generous gift from Dr. G. Dianov (University of Oxford). Purified Neil1 was kindly donated by Dr. D.O. Zharkov (ICBFM, Novosibirsk). Oligonucleotides contained non-nucleotide moieties were kindly donated by Dr. D.V. Pyshnyi (ICBFM, Novosibirsk). Oligonucleotides containing THF and 5-oxoC residues were purchased from Eurogentec (Seraing, Belgium).

The sequences of the oligonucleotides used in the experiments were as follows:

Upstream 21-mer
5′-gtggcgcggagacttagagaa-3′
Downstream 19-mer
5′-pTHF-tttggcgcggggaattcc-3′
40-mer DNA-template
5′-ggaattccccgcgccaaatttctctaagtctccgcgccac-3′
42-mer-THF
5′-agaaacaacagcactactgtactcatg**THF**attctattccagca-3′
42-mer-5oxoC
5′-agaaacaacagcactactgtactcatg**5oxoC**attctattccagca-3′
42-mer complementary
5′-tgctggaatagaattcatgagtacagtagtgctgttgtttct-3′

THF-DNA	5′-ggaagaccctgacgtt**THF**cccaacttaatcgcc-3′
	3′-ccttctgggactgcaa c gggttgaattagcgg-5′
DD-DNA	5′-ggaagaccctgacgtt**DD**cccaacttaatcgcc-3′
	3′-ccttctgggactgcaa c gggttgaattagcgg-5′
DEG-DNA	5′-ggaagaccctgacgtt**DEG**cccaacttaatcgcc-3′
	3′-ccttctgggactgcaa c gggttgaattagcgg-5′

where THF designates the 3-hydroxy-2(hydroxymethyl)-tetrahydrofuran moiety; DD designates 10-hydroxydecyl (or decanediol); and DEG designates bis-ethylene glycol (or diethyleneglycol) moieties (see Fig. 4 for structure).

2.2. Radioactive labeling of oligonucleotides

Prior to enzymatic assays, oligonucleotides were either 5′-end labeled by T4 polynucleotide kinase (New England Biolabs, OZYME, France) in the presence of [γ-^{32}P]-ATP (3000 Ci/mmol) (PerkinElmer) as described [21], or 3′-end labeled by terminal deoxynucleotidyl transferase (TdT) (New England Biolabs) in the presence of [α-^{32}P]-3′-dATP (Cordycepin 5′-triphosphate, 5000 Ci/mmol) (PerkinElmer) as recommended by the manufacturers. In addition, the 32-mer oligonucleotide containing THF was labeled by extending its 3′-end by α-^{32}P-labeled dCMP using the activity of Polβ. Unreacted [γ-^{32}P]-ATP or [α-^{32}P]-cordycepin (or cytosine) triphosphate were removed by passing the mixture over a MicroSpin^{TM} G-25 column (Amersham, USA) as recommended by the manufacturers. Complementary oligodeoxynucleotides were annealed in equimolar amounts by heating a solution to 95 °C for 3 min, followed by slow cooling to room temperature.

The 19-mer downstream oligonucleotide containing 5′-pTHF was 5′-[^{32}P]-labeled by utilizing the exchange reaction catalyzed by T4 polynucleotide kinase in the presence of [γ-^{32}P]-ATP and ADP [21]. This oligonucleotide and the upstream 21-mer oligonucleotide were annealed with the complementary 40-mer DNA-template followed by ligation by T4 DNA ligase overnight at 16 °C.

2.3. Tdp1 cleavage assays

Standard reaction mixtures (10 μl) contained 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 10 nM [^{32}P]-labeled DNA substrate and 100 nM Tdp1. After adding Tdp1 the reaction mixtures were incubated at 37 °C for 30 min. The reactions were then terminated by adding formamide dye and the mixtures were heated for 3 min at 90 °C. The products were analyzed by electrophoresis in 20% polyacrylamide gel with 8 M urea followed by autoradiography [21].

2.4. DNA repair reconstitution assay

The reaction mixture (10 μl) contained 10 nM labeled substrate in a buffer containing 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, and 10 μM dGTP. Different combinations of Tdp1 (100 nM), PNKP (20 nM), Polβ (100 nM), and FEN1 (200 nM) were added as required. The mixtures were incubated at 37 °C for 30 min and analyzed as above.

3. Results and discussion

3.1. Tdp1 produces a 3′-phosphate terminus when cleaving THF-containing DNA

Previously, we showed that human Tdp1 hydrolyzes DNA substrates with a natural AP site producing a one-nucleotide gap with 3′- and 5′-phosphates [10,11]. Tdp1 was also able to cleave synthetic analog of the AP site – THF – in contrast to enzymes possessing AP-lyase activity (e.g. Neil1 and EndoIII). Therefore it was proposed that Tdp1 acts by different mechanism compared to AP-lyase activity with the subsequent removal of the 3′-α,β-unsaturated aldehyde moiety to produce 3′-phosphate. It is quite possible that Tdp1 catalyzes the cleavage of the phosphodiester bond producing SSB with 3′-phosphate and 5′-deoxyribose termini and then that the 5′-deoxyribose is hydrolyzed spontaneously due to instability of this moiety in the absence of the 5′-terminal phosphate.

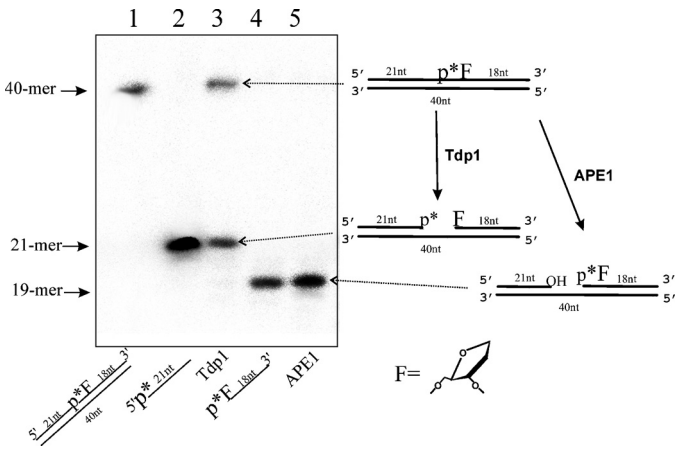

Fig. 1. Tdp1 cleaves DNA containing ^{32}P in the inner 5′ position adjacent to THF residue generating a break with the 3′-^{32}P terminus. Lane 1 shows the 40-mer product with radioactive phosphate inside the DNA after ligation upstream and radioactive labeled downstream primers. Lane 2 presents the 5′-[^{32}P]-labeled 21-mer upstream primer. The 21-mer product with the 3′-radioactive phosphate was generated by incubation of the 40-mer THF-DNA with Tdp1 (lane 3). Lane 4 shows the 19-mer downstream primer with 5′-[^{32}P]THF. The 19-mer product with the 5′-radioactive phosphate was generated by incubation of the 40-mer THF-DNA with APE1 (lane 5).

To further elucidate the mechanism whereby Tdp1 acts, a DNA duplex containing a THF moiety in one of the strands (THF-DNA) was treated with Tdp1 or APE1. The THF group in DNA is relatively stable and is not degraded spontaneously after cleavage in contrast to a natural AP site. To demonstrate that Tdp1 catalyzes the reaction of THF cleavage to generate breaks with 3′-phosphate termini, we used an oligonucleotide duplex with a radioactive phosphate inside the DNA strand placed in the 5′ adjacent position to the THF residue. This DNA was incubated with Tdp1 or APE1 and the reaction products were separated by gel electrophoresis (Fig. 1). [^{32}P]-labeled fragments of 21 and 19 nt in length can be considered for Tdp1 and APE1 cleavage, respectively. Indeed, the mobility of the product produced by Tdp1 is the same as that of the 5′-labeled upstream primer of 21 nt in length (Fig. 1, lanes 2 and 3) whereas the APE1 cleavage product shows the same electrophoretic mobility as the 5′-labeled downstream primer of 19 nt in length (Fig. 1, lanes 4 and 5). These data indicate that the cleavage of THF-DNA by Tdp1 results in the production of the cleavage fragments with a 3′-^{32}P terminus and probably a 5′-THF group in contrast to APE1 that produces the cleavage fragment with a 5′-^{32}P-THF group.

3.2. Tdp1 generates a non-phosphorylated 5′-THF-terminus by the cleavage of THF-containing DNA

To examine the events on the 5′-end of the AP site processed by Tdp1, the 3′-end labeled DNA duplexes were used (Fig. 2a). The 42-mer containing THF was first extended using [α-^{32}P]-3′-deoxyadenosine (cordycepin) triphosphate and the activity of TdT that resulted in a 43-mer used as top strand. The THF containing DNA duplex was treated with APE1 and Tdp1 (Fig. 2a, lanes 1 and 3, respectively). Tdp1 cleaves such DNA producing two fragments with different mobilities (Fig. 2a, lane 3). One band is the 42-mer resulting from removal of the 3′-cordycepin moiety due to Tdp1 nucleosidase activity leaving behind a 3′-^{32}P moiety [22]. The band with high mobility can be attributed to the product of double digestion of the DNA strand by Tdp1 – THF hydrolysis and the removal of 3′-cordycepin. We also used a DNA strand of the same sequence as THF-DNA but containing a 5-oxoC residue (5-oxoC-DNA) that can be processed by the bifunctional DNA glycosylase Neil1 in contrast

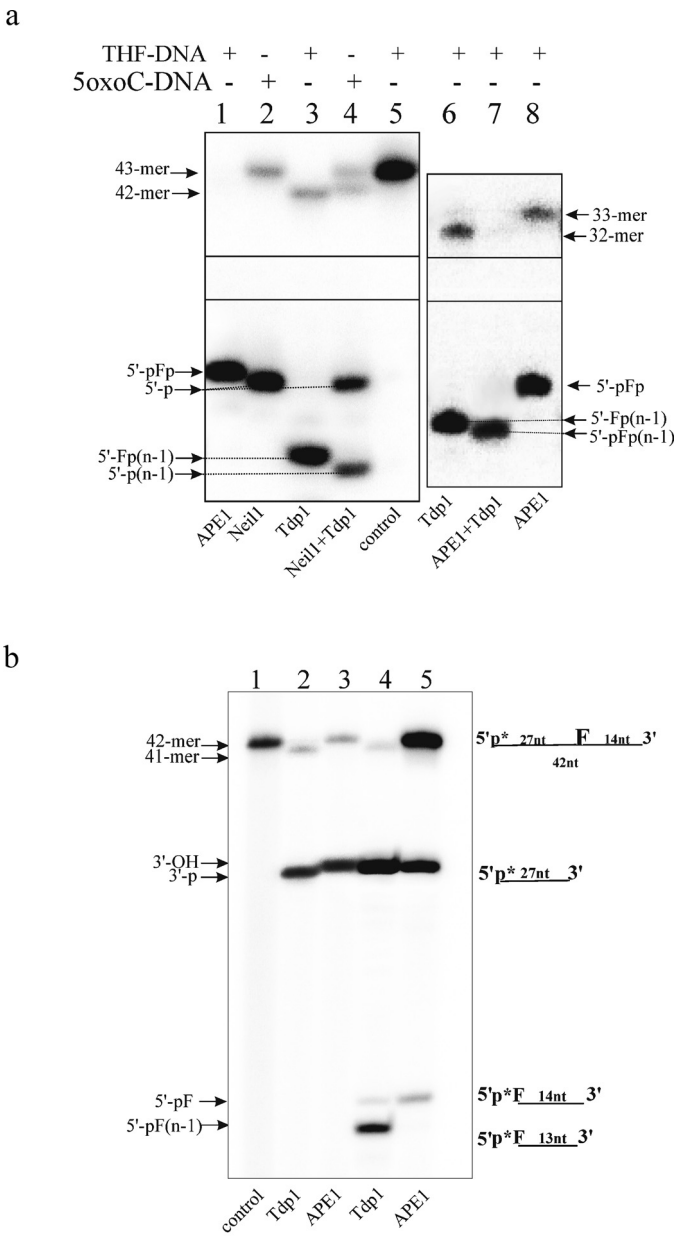

Fig. 2. Tdp1 catalyzes the cleavage of THF-containing DNA to generate a break with a 5′-THF terminus. (a) Cleavage of the 3′-end labeled THF-DNA by Tdp1. The 3′-[^{32}P]-labeled DNA containing THF or 5-oxoC was incubated with different enzymes. Arrows indicate the mobility of different products. Lane 5 shows the initial substrate, the 43-mer DNA duplex. (b) Tdp1 creates non phosphorylated 5′-termini and the 3′-phosphate termini. Lane 1 shows the initial substrate, the 5′-[^{32}P]-labeled THF-containing 42-mer. Lane 2 shows the product with the 3′-P after incubation of the 5′-[^{32}P]-labeled THF-containing 42-mer with Tdp1. Lane 3 presents the product with the 3′-OH after incubation of the 5′-[^{32}P]-THF-DNA with APE1. Products of hydrolysis of the THF-containing 42-mer by Tdp1 (lane 4) or by APE1 (lane 5) followed by phosphorylation using T4 polynucleotide kinase and [γ-^{32}P]-ATP.

to the THF residue that is resistant to the activity of this enzyme. After hydrolysis of the 5-oxoC-DNA by Neil1, the product with a 5′-P group in the break is generated (Fig. 2a, lane 2) that moves in the gel faster than the product of THF-DNA cleavage by APE1 bearing a 5′-furanophosphate group (lane 1). Addition of Tdp1 in the reaction mixture after hydrolysis by Neil1 of 5-oxoC-DNA results in the removal of 3′-cordycepin by Tdp1. One can see that the final product (5′-P($n-1$)) (lane 4) migrates in the gel slightly faster than the product of THF-DNA cleavage by Tdp1. Furthermore, when the

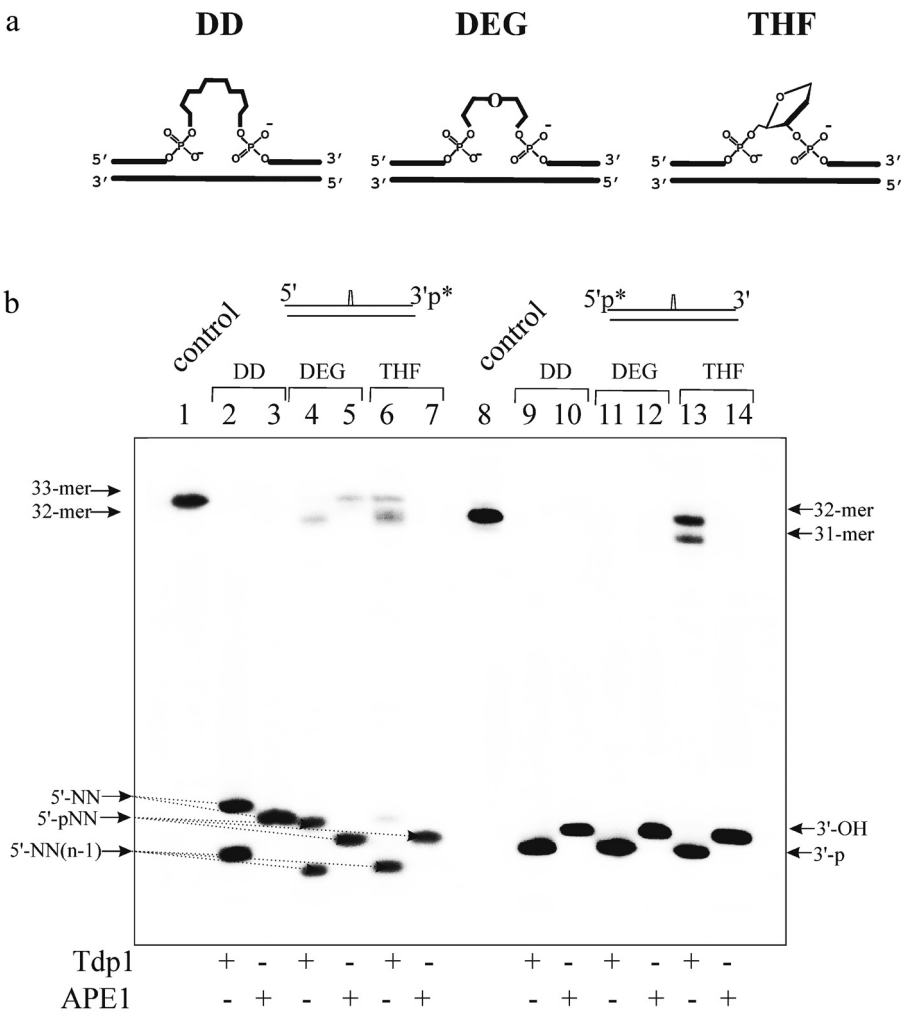

Fig. 3. Tdp1 cleaves DNA containing synthetic analogs of the AP site–non-nucleotide (NN) insertions. (a) Schematic view of the NN insertions in DNA structures used in the experiments. (b) Defined amounts of Tdp1 (100 nM) or APE1 (100 nM) were incubated for 30 min with 10 nM 3′-[^{32}P]-labeled DNA duplex (lanes 1–7) or 5′-[^{32}P]-labeled DNA duplex (lanes 8–14) containing the NN moieties. Lanes 1 and 8 correspond to the initial DNA substrates. Arrows indicate the mobility of different products.

3′-end labeled 33-mer THF-DNA (Fig. 2a, right panel) hydrolyzed by APE1 (lane 8) was additionally treated by Tdp1 that removes the 3′-nucleoside residue, the final product (5′-pF(n – 1)p) (lane 7) migrates in the gel more slowly than the product of Tdp1 cleavage (lane 6) as expected.

For further evidence that Tdp1 creates non-phosphorylated 5′-termini, an unlabeled THF-containing 42-mer was cleaved by Tdp1 or APE1 and the resulting fragments were phosphorylated by T4 polynucleotide kinase using [γ-^{32}P]-ATP (Fig. 2b). One can see that DNA fragments of both 27 and 14 nt in length produced by Tdp1 were effectively 5′-[^{32}P]-labeled (Fig. 2b, lane 4) whereas the levels of the labeled products generated by APE1 were different (lane 5): the 5′-OH fragment of 27 nt was labeled with the much higher efficiency than the 5′-pTHF fragment of 15 nt that can be labeled by the exchange phosphorylation. T4 PNK also possesses 3′-phosphatase activity [23] that removes the 3′-phosphate group created by Tdp1. Therefore, the electrophoretic mobility of the 27 nt fragment produced by Tdp1 (lane 4) has the same mobility as the one observed in the case of the fragment generated by APE1 (lane 5) whereas cleavage of previously the 5′-[^{32}P]-labeled DNA results in higher mobility of the Tdp1 product (lane 2) in comparison to the APE1 cleavage product (lane 3). The minor band observed after cleavage of the THF-containing 42-mer by Tdp1 (lane 4) belongs most likely to the 15-mer with the 3′-terminal nucleoside residue.

3.3. Tdp1 can cleave DNA containing non-nucleotide insertions

Next, we tested the ability of Tdp1 to cleave DNA containing another synthetic analogs of AP site, e.g. non-nucleotide insertions, decanediol (DD) and diethyleneglycol (DEG) moieties. It was shown that these moieties can be substrates of APE1 [24]; therefore it was of interest to analyze the action of Tdp1 on these artificial insertions in comparison to THF and natural AP site. For these experiments we used 5′- or 3′-[^{32}P]-labeled 32/33-mer DNA duplexes. As shown in Fig. 3, Tdp1 catalyzes the hydrolysis of DD- and DEG-containing DNA even more effectively than THF-containing DNA particularly in the case of DD-containing DNA (compare lanes 2, 4, 9, and 11 with lanes 6 and 13) generating DNA breaks with 3′-P and 5′-DD or 5′-DEG termini. Therefore, the cleavage products in the case of non-nucleotide insertions are the same as in the case of THF. They have 3′-phosphate and 5′-substitution margins. It was shown earlier that these insertions in DNA influence the geometry of the DNA helix by inducing DNA bending and reduced duplex stability [25,26]. It should be noted that the DD moiety induces

more remarkable changes in the DNA duplex than the DEG and THF moieties [25]. A natural AP site is also known to induce a local distortion of the DNA duplex followed by its bending [27]. Therefore, one may suggest that an AP site or its synthetic analogs followed by a downstream DNA fragment are recognized and processed by Tdp1 as is the modification linked to the 3′-phosphate including adducts formed by Top1 or by the Tdp1 H493R mutant (SCAN1) [4,7–9].

3.4. Repair of 5′-THF and other AP site analogs can be processed by the long-patch BER pathway

The repair of DNA structures containing a DD or DEG moiety as the synthetic analog of the AP site was analyzed in a minimal reconstituted BER system consisting of purified proteins (Fig. 4). The 5′ ^{32}P-labeled 32-mer DNA duplex containing a DD or DEG moiety in position 16 was incubated with purified recombinant Tdp1, PNKP, Pol β, FEN1 and dNTP to mimic the long patch DNA repair system. The reaction mixture containing Tdp1 but lacking PNKP (Fig. 4, lanes 2 and 7) generated a product with a 3′-phosphate. Addition of PNKP resulted in a 15-mer product with a 3′-OH terminus (Fig. 4, lanes 3 and 8). Finally, Polβ replaces the missing DNA segment by dGMP (Fig. 4, lanes 4 and 9) or catalyzes strand displacement synthesis after gap filling in the presence of all dNTPs (Fig. 4, lanes 5 and 10). Addition of FEN1 to the reaction mixture stimulated strand-displacement DNA synthesis by Pol β (lanes 6 and 11). Therefore, the repair of non-nucleotide insertions in DNA initiated by Tdp1 can be processed by long patch BER to fully restore the intact DNA and generate the products of the expected lengths at each intermediate stage of the repair process.

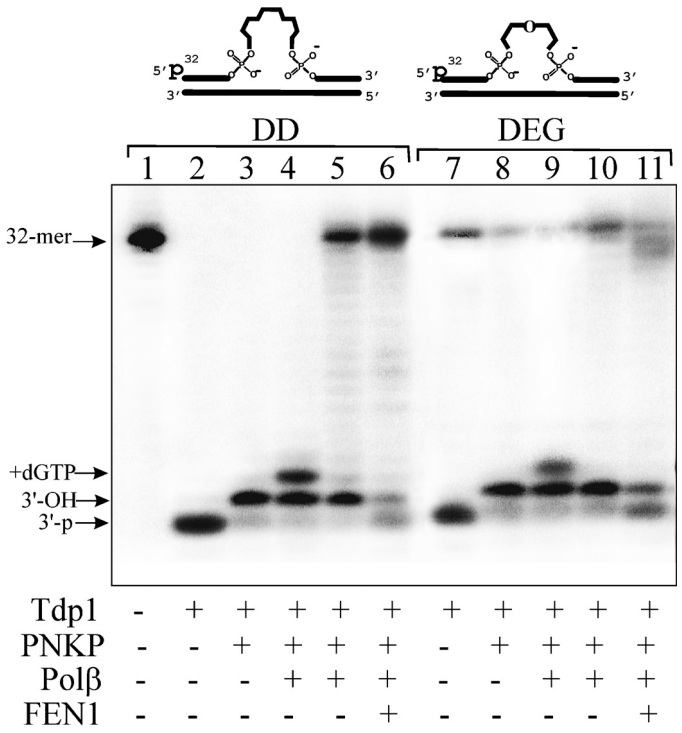

Fig. 4. In vitro reconstitution of the Tdp1-initiated repair of DNA containing a DD or DEG moiety. The 5′-end labeled DNA duplex containing DD (lanes 1–6) or DEG (lanes 7–11) was subsequently incubated with Tdp1 (lanes 2 and 7), PNKP (lanes 3 and 8), Pol β in the presence of dGTP (lanes 4 and 9) or all of dNTPs (lanes 5 and 10), FEN1 (lanes 6 and 11) to monitor strand displacement DNA synthesis. The components present in the different reaction mixtures are indicated.

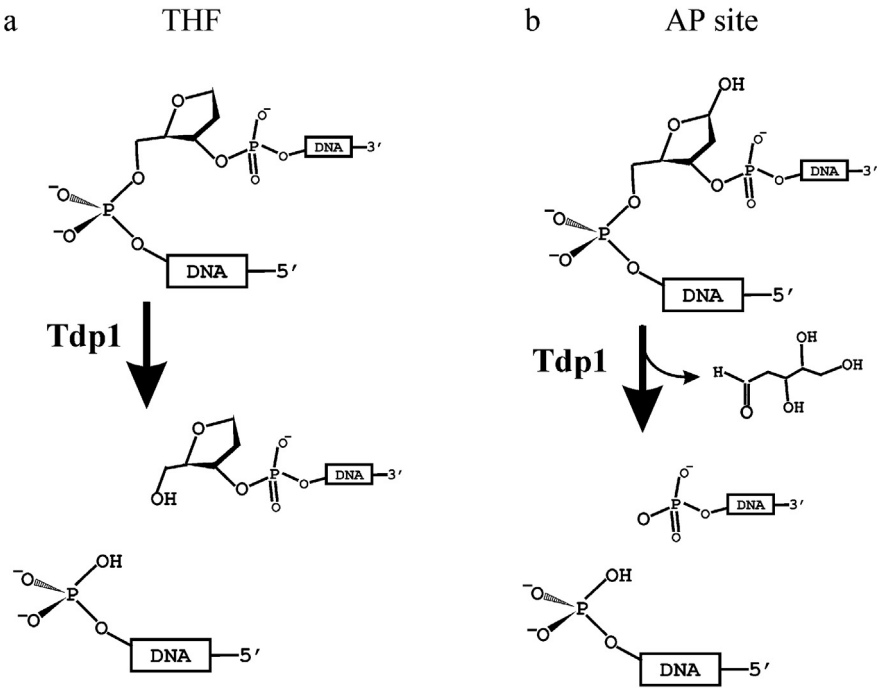

Fig. 5. Scheme of the hydrolysis of the AP site or its synthetic analogs in DNA by Tdp1. (a) Tdp1 catalyzes the cleavage of DNA containing THF or other synthetic analogs of the AP site to generate a break with the 5′-analog residue and the 3′-phosphate termini. (b) In the case of a natural AP site, 5′-deoxyribose is probably hydrolyzed spontaneously due to its instability.

4. Conclusions

In summary, our results demonstrate that Tdp1 catalyzes cleavage of DNA containing THF or other analogs of the AP site to generate breaks with the 5′-analog residue and the 3′-phosphate termini (Fig. 5a). In the case of a natural AP site, 5′-deoxyribose is probably hydrolyzed spontaneously due to its instability and the final product has 3′- and 5′-phosphates in the break (Fig. 5b). According to our observation 5′-deoxyribose is extremely labile and degraded faster than added $NaBH_4$. Therefore the resulting DNA break can be processed by DNA polymerases lacking lyase activity or in the case of its mutation. High instability of 5′-deoxyribose produced by Tdp1 might provide an opportunity to perform DNA repair in cells deficient in 5′-dRP lyase activity [28,29]. In contrast, the 5′-dRP moiety generated by APE1 is stable and the following processing of break needs DNA polymerase containing lyase activity. Moreover Tdp1 can process effectively AP sites located in ssDNA or inside cluster of DNA damages [10,11]. Tdp1 can promote its activity without Mg^{2+} while APE1 activity depends on Mg^{2+}. Therefore TDP1 can perform AP-site cleavage under conditions which are different from APE1 cleavage activity but might be important for specific DNA structures and repair pathways.

Conflict of interest

None.

Acknowledgements

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