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AP-site cleavage activity of tyrosyl-DNA phosphodiesterase 1

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ABSTRACT

APE-independent base excision repair (BER) pathway plays an important role in the regulation of DNA repair mechanisms. In this study it has been found that recently discovered tyrosyl-DNA phosphodiesterase 1 (Tdp1) catalyzes the AP site cleavage reaction to generate breaks with the 3′- and 5′-phosphate termini. The removal of the 3′-phosphate is performed by polynucleotide kinase phosphatase (PNKP). Tdp1 is known to interact stably with BER proteins: DNA polymerase beta (Pol β), XRCC1, PARP1 and DNA ligase III. The data suggest a role of Tdp1 in the new APE-independent BER pathway in mammals.
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1. Introduction

Tyrosyl-DNA phosphodiesterase (Tdp1) was discovered as an enzymatic activity from *Saccharomyces cerevisiae* that specifically hydrolyzes the phosphodiester linkage between the O-4 atom of a tyrosine and a DNA 3′ phosphate [1]. This type of linkage is typical for the covalent reaction intermediate produced upon Topoisomerase 1 cleavage of one DNA strand. Human Tdp1 can also hydrolyze other 3′-end DNA alterations that are covalently linked to the DNA, indicating that it may function as a general 3′-DNA phosphodiesterase and repair enzyme [2]. Oxidative damage at DNA ends (i.e. the termini of DNA single- or double-strand breaks) or intermediates in the base excision repair (BER) process may represent substrates for Tdp1 in vivo. For example, it is conceivable that Tdp1 acts on the 3′ phospho α, β unsaturated aldehyde (3′ dRP) that results from β-elimination by the base-specific mammalian DNA glycosylases/AP lyases (for example, OGG1 or NTH1) [3,4]. Also Tdp1 can remove the tetrahydrofuran moiety from the 3′-end of DNA [5].

Human Tdp1 always cleaves at the most 3′ backbone phosphate to remove one nucleoside from either DNA or RNA and leave a 3′ phosphate terminus. The yeast enzyme was reported to be unable to cleave the DNA phosphate backbone, but it is possible that the

assay used was not sensitive enough to detect this activity [6]. The 3′-phosphate-end generated by Tdp1 has to be hydrolyzed to a 3′-hydroxyl in order to enable the following DNA repair. Polynucleotide kinase phosphatase (PNKP), a bifunctional enzyme with 5′-kinase and 3′-phosphatase activities, has been suggested as a reasonable candidate in human cells for the repair of these 3′-phosphate lesions. Tdp1 has been shown to exist in complex with PNKP in human cells [7,8].

Since several thousand apurinic/apyrimidinic (abasic or AP) sites are generated spontaneously every day in a living cell [3,9] and this structure is the key intermediate of BER pathway, it was of particular interest to find out whether Tdp1 can hydrolyze of AP site. It is known that the first step in BER, excision of damaged base, is catalyzed by DNA glycosylases, many of which are monofunctional and hydrolyze *N*-glycosidic bonds to generate abasic sites. The major enzyme of eukaryotic cells catalyzing the endonuclease cleavage of AP sites is apurinic/apyrimidinic endonuclease 1 (APE1) that cleaves the phosphodiester bond at the 5′ end of abasic site. Bifunctional DNA glycosylases that excise oxidized bases also possess an intrinsic lyase activity, cleaving the DNA at the resultant AP sites [10]. These DNA glycosylase/AP lyases belong to two broad classes of enzymes, based on their reaction mechanism. *Escherichia coli* Nth is the representative of one class that utilizes an internal lysine as the active site nucleophile and cleaves the DNA strand at the AP site by β elimination, generating a 3′ phospho α,β-unsaturated aldehyde (3′-dRP) at the strand break [11]. In contrast, another class of mammalian DNA glycosylases, belonging to the family of *E. coli* Nei and Fpg (named Nei-like (NEIL)) catalyzes βδ elimination at the AP site and

removes the deoxyribose residue to produce a 3′ phosphate terminus at the DNA strand break [12].

In this report we show that Tdp1 generates DNA strand break with the 3′ phosphate termini from the abasic (AP) site therefore it can function in a fashion similar to NEIL1 in the APE-independent BER pathway.

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); phage T4 polynucleotide kinase was purchased from Biosan (Russia); stained molecular mass markers were from Fermentas (Lithuania), reagents for electrophoresis and buffer components from Sigma (USA). Ultrapure dNTPs were from SibEnzyme (Russia).

The recombinant Tdp1 was purified to homogeneity as described [13] with the additional purification steps using plasmid pET 16B-Tdp1 kindly provided by Dr. K.W. Caldecott (University of Sussex, United Kingdom). Purification protocol and coomassie stained protein gel are shown in Supplementary data. The recombinant purified UDG, DNA polymerase β, APE1 and XRCC1 were a generous gift from Dr. S.N. Khodyreva (ICBFM, Novosibirsk). Phage T4 endonuclease III, NEIL1, PNKP and DNA ligase III were kindly donated by Dr. D.O. Zharkov (ICBFM, Novosibirsk).

2.2. Radioactive labeling of oligonucleotides

Oligodeoxynucleotides were 5′-[^{32}P]-labeled with T4 polynucleotide kinase and [γ^{32}P] ATP as described [14]. Unreacted [γ^{32}P] ATP was removed by passing the mixture over a MicroSpin™ G-25 column (Amersham, USA) using the manufacturer's suggested protocol. 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 sequences of the oligonucleotides used in experiments are shown:

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AP-DNA:   5′-GGCGATTAAGTTGGGUAACGTCAGGGTCTTCC-3′
          3′-CCGCTAATTCAACCC   GTTGCAGTCCCAGAAGG-5′
THF-DNA:  5′-GGCGATTAAGTTGGGTHFAACGTCAGGGTCTTCC-3′
          3′-CCGCTAATTCAACCC G   TTGCAGTCCCAGAAGG-5′
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2.3. Endonuclease assays

Standard reaction mixtures (10 μl) contained 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 5 mM MgCl$_2$, 10 nM 5′-[^{32}P]-labeled DNA substrate and necessary enzymes (Tdp1, NEIL1, APE1 or EndoIII). For the preparation of natural AP site, a AP-DNA duplex was first incubated in reaction buffer with UDG (0.5 U/μl) for 15 min at 37 °C. The reaction mixtures were incubated at 37 °C for 30 min. Then reactions were terminated by adding of the 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 [14].

2.4. DNA repair reconstitution assay

The reaction mixture (10 μl) contained 10 nM of the labeled substrate in a buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM MgCl$_2$, 0.5 mM dCTP, 1 mM ATP. Different combinations of Tdp1 (100 nM), DNA polymerase beta (Pol β) (50 nM), PNKP (300 nM), and DNA ligase III (10 nM) were added as required. The mixtures were incubated at 37 °C for 30 min and analyzed as above.

3. Results and discussion

To identify a new activity of Tdp1, we analyzed the capability of enzyme to cleave DNA structure containing a natural abasic site. The 32 mer DNA duplex bearing a single uridine moiety in the 5′ end-labeled strand was treated with UDG to produce AP site that was efficiently cleaved by Tdp1 (Fig. 1A, lane 1). Without addition of UDG, DNA duplex with uridine residue was not cleaved by Tdp1. This activity was compared with the action of bifunctional DNA glycosylase NEIL1 (lane 2) as well as with activity of two endonucleases, APE1 and T4 endonuclease III (EndoIII) (lanes 3 and 4, respectively). AP site cleavage mechanisms catalyzed by Endo III, APE1, and NEIL1 are shown schematically in Fig. 1B. One can see from the experimental data (Fig. 1A, lanes 1 and 2) that the mobility of the product of cleavage produced by Tdp1 was the same as generated by AP lyase activity of NEIL1, which creates the 3′ terminal phosphate (3′-P) in the AP site cleavage reaction. Both APE1 and EndoIII cleavage products bearing the 3′-OH and the 3′-dRP groups, respectively, demonstrate the lower mobility in gel electrophoresis (lanes 3 and 4). This data point out that, Tdp1 cleaves AP-DNA generating the 3′-P termini.

We also tested Tdp1 activity towards AP sites located in ssDNA or dsDNA in comparison with the same DNA structures containing an inside strand tetrahydrofuran (THF) moiety as a synthetic analogue of abasic site (Fig. 2). Tdp1 is more active in the cleavage of ssDNA containing THF moiety (Fig. 2, lane 2) or natural AP site (lane 9) in comparison with dsDNA containing THF or natural AP site in one of the strands (lanes 5 and 12, respectively). APE1 shows preferentially hydrolysis of the AP site or its synthetic analogue located in one strand of dsDNA (compare lanes 4 and 11 with lanes 1 and 8). Unlike Tdp1 NEIL1 was unable to cleave DNA with THF moiety (lanes 3 and 6), but catalyzed the hydrolysis of both ss and dsDNA containing natural AP site (lanes 10 and 13). It should be noted that the experiments with Tdp1 also showed the product P1. This indicates that Tdp1 was able to remove a 3′ terminal mononucleoside moiety in both ssDNA and DNA duplex to produce 3′-phosphate whereas resulting DNA containing 3′-phosphate
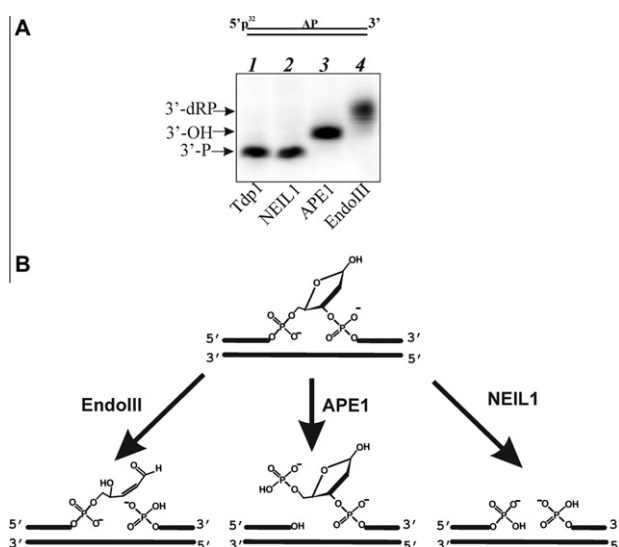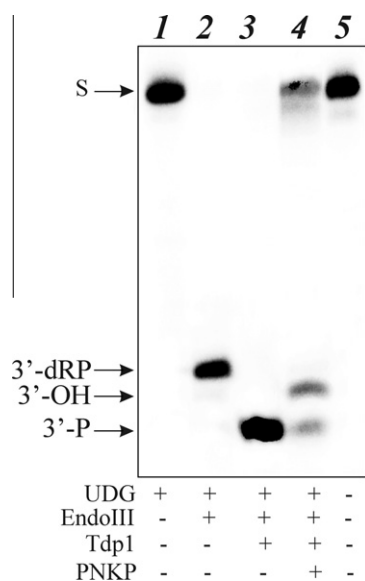

Fig. 1. Tdp1 is able to cleave 5′-end labeled AP-DNA structure. (A) The 15mer product with 3′-phospate (3′-P) was generated by incubation of the 5′-end labeled AP-DNA with Tdp1 (lane 1). The same product with the 3′-P was observed after incubation with NEIL1 (lane 2). Lane 3 shows the 15mer product with the 3′-OH after incubating AP-DNA with APE1 and lane 4 shows the product with 3′-dRP after hydrolysis of AP-DNA with EndoIII. (B) Scheme of hydrolysis of the AP site in one strand of DNA duplex by different enzymes.
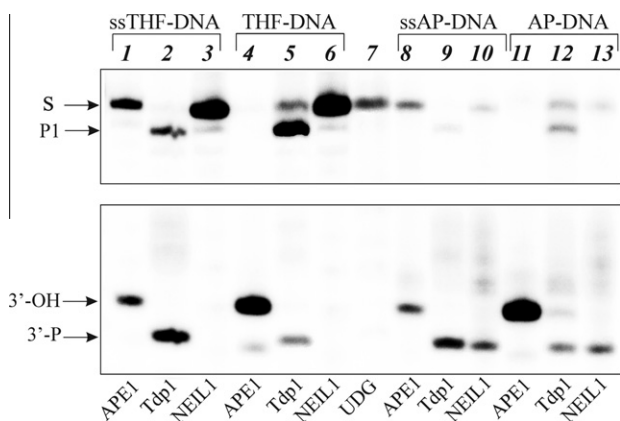
Fig. 2. Comparative efficiency of the hydrolysis of AP/THF sites in single and double stranded DNAs. Defined amounts of Tdp1 (100 nM), NEIL1 (100 nM) or APE1 (10 nM) were incubated for 30 min with 10 nM ssDNA substrate (lanes 1–3, 8–9) and dsDNA duplex (lanes 4–6, 11–13) containing the THF-moiety (lanes 1–6) or natural AP site (lanes 8–13). Lane 7 corresponds to the AP-DNA. Arrows indicate the mobility of different products. S denotes the initial substrate, the 32mer DNA duplex.

group was not further processed by Tdp1. This indicates that Tdp1 can not act as a 3′ exonuclease or 3′ phosphatase [5].

Tdp1 was shown earlier is able to remove a tetrahydrofuran moiety from the 3′-end of short ssDNA [5]. To analyze the Tdp1 capacity to remove 3′-dRP moiety in DNA single strand break (SSB), we incubated Tdp1 with 32-mer AP-DNA pretreated by EndoIII. Endo III cleaves AP site generating SSB containing the 3′ terminal dRP moiety (Fig. 3, lane 2). Finally, 15-mer product was observed (lane 3). Therefore, Tdp1 can operate efficiently with the SSB 3′-end to remove dRP moiety after hydrolysis of phosphodiester bond at the 5′-side of AP site. This leads most likely to the production of the 3′-phosphate. The 3′-end of the resulting gap requires further processing to generate a 3′-hydroxyl group that could be used as substrate of DNA polymerases in the following BER steps; such processing has previously been shown to involve
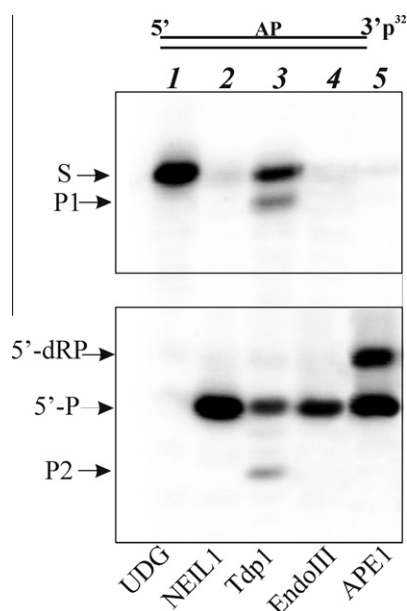
PNKP [4]. To verify the role of PNKP in this reaction, PNKP was added into reaction mixture after Tdp1. The mobility of the resulting product was changed, probably as the consequence of action of the 3′ phosphatase activity of PNKP removing the 3′-phosphate (Fig. 3). After this treatment Pol β is able to introduce dNMP moiety into the 3′-end of oligonucleotide (see below).

We also utilized the 3′-end labeled AP-DNA as a substrate to examine the 5′-end of AP site processing by Tdp1 (Fig. 4). The top strand was labeled by the extending of its 3′-end by α-^{32}P-labeled dCMP using activity of Pol β. This resulted in a 33-nt top strand. After UDG treatment of the labeled 33-mer (lane 1), Tdp1 was added to the reaction. The resulting major product (lane 3) had the same mobility as compared to NEIL1 and EndoIII cleavage products (lanes 2 and 4, respectively) that are known to generate the 5′-phosphate. Therefore, it is reasonable to suggest that Tdp1 generates a DNA break with the 5′-phosphate (5′-P) terminus. In the case of treatment with APE1 two bands of products were observed (lane 5) corresponding to the 5′-dRP-containing fragment (the upper band) and the product of the spontaneous hydrolysis of the deoxyribose resulting in the 5′-P terminus. It should be noted that two additional bands of products (P1 and P2) are formed in the Tdp1 catalyzed reaction (lane 3). One of them (P1) can be attributed to the removal of one nucleoside from the 5′-end by Tdp1, similar to the activity against the 5′ phosphotyrosyl substrates reported for the yeast Tdp1 [15]. The second band P2 migrates slightly faster than the 17-mer major product and can be suggested to be a product of P1 cleavage by Tdp1. In summary, our results demonstrate that Tdp1 protein after cleavage of the AP site-containing DNA duplex is able to generate one window gap with the 5′-phospate and the 3′-phosphate termini.

We analyzed the repair of AP site in a minimal reconstituted BER system consisting of purified proteins (Fig. 5). The 5′ ^{32}P-labeled 32-mer DNA duplex containing uridine at the position 16 was incubated with the purified recombinant UDG, Tdp1, PNKP, Pol β, DNA ligase III, and XRCC1 to mimic DNA repair system. The reaction mixture containing Tdp1 but lacking PNKP (Fig. 5, lane 2) generated a product with a 3′-phosphate, which is identical to that produced by NEIL1. Addition of PNKP and XRCC1 resulted in a 15-mer product

Fig. 3. Tdp1 activity on DNA substrate with 3′-dRP moiety in the single strand break. The 15-mer with 3′-dRP was generated by incubating 10 nM AP-DNA with EndoIII (100 nM) (lane 2). Following incubation of this product with Tdp1 (100 nM) results in the 15-mer with the 3′-P (lane 3). Lane 4 shows the 15-mer product with the 3′-OH after adding PNKP in the reaction mixture (300 nM). Lane 1 corresponds to the AP-DNA Lane 5 – DNA not treated with UDG.

Fig. 4. Cleavage of the 3′-end labeled AP-DNA structure by Tdp1. The 3′-end labeled AP-DNA was incubated with NEIL1 (lane 2), Tdp1 (lane 3), Endo III (lane 4) and APE1 (lane5). Arrows indicate the mobility of different species of products. Lane 1 corresponds to the AP-DNA.
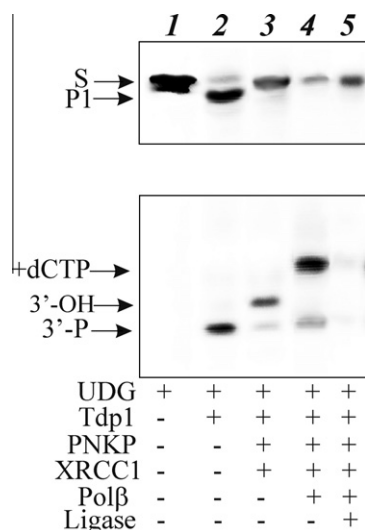
Fig. 5. Reconstitution of the AP-DNA substrate repair initiated by Tdp1. The 5′-end labeled AP-DNA substrate was subsequently incubated with the UDG (lane 1), Tdp1 (lane 2), PNKP and XRCC1 (lane 3), Pol β (lane 4), DNA ligase III (lane 5) to monitor DNA repair. The components present in different reaction mixtures are indicated.

with the 3′-OH terminus (lane 3). XRCC1, a scaffold protein that normally couples the action of Tdp1 with the other BER proteins, such as DNA polymerase β, PNKP, DNA ligase III, and poly(ADP-ribose)polymerase 1 (PARP1) increased the efficiency of the 3′-hydroxyl generation by PNKP [2]. The interaction of XRCC1 with PNKP has been shown to stimulate both the 5′-kinase and 3′-phosphatase activities of this enzyme [16]. Besides the hydrolysis of the 3′-phosphate end, PNKP catalyzes the phosphorylation of the 5′-end of the DNA (lane 3). Lastly, DNA polymerase β replaces the missing DNA segment (lane 4) and DNA ligase reseals the DNA (lane 5). So, the repair of AP site initiated by Tdp1 fully restored the intact DNA and generated the products of the expected lengths at each intermediate stage. In summary, the results presented here show that the human Tdp1 protein can initiate repair of AP sites and 3′-dRP termini predict that Tdp1/PNKP could take over repair initiated by some DNA glycosylases. PNKP and Pol β play key roles in this repair pathway however the both enzyme activities are stimulated by XRCC1.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.01.032.

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