DNA Repair 11 (2012) 53–64

Contents lists available at SciVerse ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

Trypanosoma brucei AP endonuclease 1 has a major role in the repair of abasic sites and protection against DNA-damaging agents

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ARTICLE INFO

Article history:
Received 2 August 2011
Received in revised form 7 October 2011
Accepted 7 October 2011
Available online 29 October 2011

Keywords:
AP site
Strand break
AP endonuclease
APE1
BER
Trypanosoma brucei

ABSTRACT

DNA repair mechanisms guarantee the maintenance of genome integrity, which is critical for cell viability and proliferation in all organisms. As part of the cellular defenses to DNA damage, apurinic/apyrimidinic (AP) endonucleases repair the abasic sites produced by spontaneous hydrolysis, oxidative or alkylation base damage and during base excision repair (BER). *Trypanosoma brucei*, the protozoan pathogen responsible of human sleeping sickness, has a class II AP endonuclease (TBAPE1) with a high degree of homology to human APE1 and bacterial exonuclease III. The purified recombinant enzyme cleaves AP sites and removes 3′-phosphoglycolate groups from 3′-ends. To study its cellular function, we have established TBAPE1-deficient cell lines derived from bloodstream stage trypanosomes, thus confirming that the AP endonuclease is not essential for viability in this cell type under *in vitro* culture conditions. The role of TBAPE1 in the removal of AP sites is supported by the inverse correlation between the level of AP endonuclease in the cell and the number of endogenously generated abasic sites in its genomic DNA. Furthermore, depletion of TBAPE1 renders cells hypersensitive to AP site and strand break-inducing agents such as methotrexate and phleomycin respectively but not to alkylating agents. Finally, the increased susceptibility that TBAPE1-depleted cells show to nitric oxide suggests an essential role for this DNA repair enzyme in protection against the immune defenses of the mammalian host.

1. Introduction

Abasic sites are one of the most common DNA lesions that arise in DNA and if left unrepaired, have both mutagenic and cytotoxic effects [1]. The loss of purine or pyrimidine bases in cellular DNA occurs by spontaneous hydrolysis due to the inherent lability of the N-glycosyl bond. AP sites are also generated by the action of endogenous factors such as reactive oxygen species produced by the normal metabolism of the cell or by exposure to chemical and physical exogenous agents. Moreover, in the BER pathway, damaged bases are excised by DNA glycosylases, giving rise to abasic sites [2,3]. DNA strand breaks are also a major threat to genetic stability. Strand breaks containing modified 3′-ends such as 3′-phosphoglycolate (3′-PG) and 3′-phosphate (3′-P) may arise by the attack of DNA by free radicals [4,5] or caused by the action of some antitumoral agents such as neocarzinostatin and bleomycin [6]. In addition, certain glycosylases can incise AP sites generating single-strand breaks with blocked 3′-ends. Human 8-oxoguanine-DNA glycosylase (OGG1) and the endonuclease III (NTH1) have intrinsic associated β-lyase activity that cleaves the phosphodiester backbone 3′ to the abasic site through a β-elimination reaction to produce a 3′-α, β-unsaturated aldehyde [7,8] while endonuclease VIII-like glycosylases incise the abasic site by β,∂-elimination generating a 3′-P terminus [9]. Abasic sites and blocked-ends must be removed prior to completion of repair by the coordinated actions of DNA polymerase and DNA ligase activities [10–12].

AP site repair is initiated by class II AP endonucleases, which catalyze the hydrolytic cleavage of the phosphodiester bond 5′ to the AP site [2]. There are two structurally unrelated families of class II AP endonucleases. The first family is comprised by endonuclease IV from *Escherichia coli* (endoIV) and the homologous AP endonuclease 1 (Apn1) from *Saccharomyces cerevisiae* [13]. The second family includes *E. coli* exonuclease III (exoIII) and human APE1 [14]. APE1 has a broad specificity for AP sites but can also act as a 3′-phosphodiesterase [15,16]. APE1 is the major phosphoglycolate removal activity in human cell extracts [17]. The human AP

Abbreviations: AP, apurinic/apyrimidinic; TBAPE1, *Trypanosoma brucei* AP endonuclease 1; LMAP, *Leishmania major* AP endonuclease; exoIII, exonuclease III; endoIV, endonuclease IV; OGG1, 8-oxoguanine DNA glycosylase 1; NTH1, endonuclease III; BSD, blasticidin S transferase; HYG, hygromycin phosphotransferase; F, tetrahydrofuranyl residue; 3′-PG, 3′-phosphoglycolate; 3′-P, 3′-phosphate; MXA, methoxyamine; MTX, methotrexate; PLM, phleomycin; CPT, camptothecin; MMS, methyl methanesulphonate; NO, nitric oxide; Tdp1, tyrosyl-DNA-phosphodiesterase; PNKP, polynucleotide kinase phosphatase.

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doi:10.1016/j.dnarep.2011.10.006

endonuclease also has a weak DNA 3′-phosphatase activity *in vitro*, but polynucleotide kinase phosphatase (PNKP) has been reported as the primary 3′-phosphatase activity responsible for the excision of 3′-P lesions in mammalian cells [18,19]. APE1 is also an activator of oxidized transcription factors [20]. This function relies on the cysteine 65 located at the N-terminal domain [21]. The AP endonuclease is essential in mammalian cells as mice lacking both *Ape1* alleles die during early embryonic development [22]. Recent studies involving downregulation of APE1 by RNAi in several tumour cell lines or by generation of conditional *Ape1* knockout mouse embryonic fibroblasts have revealed that cell death caused by the APE1 defect can be prevented by the independent expression of its DNA repair or redox regulatory functions [23,24].

Human cells possess a second AP endonuclease related to exoIII named APE2, which is endowed with strong 3′–5′ exonuclease and 3′-phosphodiesterase but very low AP endonuclease activity [25]. Similarly to human cells, kinetoplastid parasites have two genes that code for putative AP endonucleases of the exoIII family but lack endoIV homologs. We have previously characterized the *Leishmania major* AP endonuclease (LMAP) whose C-terminal domain shares a 38% sequence identity with APE1 [26]. Unlike the human enzyme, LMAP lacks the cysteine responsible for the redox activity. Instead, the protozoan protein has AP endonuclease and 3′-phosphodiesterase activities equally efficient and its expression in AP endonuclease-deficient *E. coli* mutants confers resistance to killing by oxidative and alkylating agents. In comparison to human APE1, the parasite AP endonuclease has subtle amino acid changes in its active site that confer a higher capacity to remove 3′-blocking ends [27]. A second putative AP endonuclease with a higher degree of homology to human APE2 has been identified in both *Leishmania* and *Trypanosoma*. However, its biochemical properties have not yet been determined and its biological role remains to be established.

Trypanosoma brucei, the African trypanosome, is the protozoan parasite transmitted by the tsetse fly vector and responsible for human African trypanosomiasis (sleeping sickness) and a related disease in livestock. During its life cycle, *T. brucei* alternates between an insect and a mammalian host (procyclic and bloodstream forms respectively) and the adaptation to the environmental conditions requires significant structural and physiological changes. Our recent work has focused on characterizing the DNA repair mechanisms employed by the parasite to counteract the deleterious effects of host-generated oxidative stress. In *T. brucei*, we have identified the corresponding APE1 ortholog, named TBAPE1 henceforth. Our study shows that TBAPE1 is a nuclear enzyme with both AP endonuclease and 3′-phosphodiesterase activities *in vitro*. To further understand how the parasite cell deals with AP sites, we have generated TBAPE1-deficient cells derived from the bloodstream form. Depletion of the TBAPE1 protein is not lethal but results in a significant increase in abasic DNA damage. The deficiency in AP endonuclease renders cells hypersensitive to methotrexate, methoxyamine, phleomycin and nitric oxide. Our findings provide the first direct evidence that TBAPE1 is the major class II AP endonuclease activity present in *T. brucei* cells and exerts protection against abasic and DNA strand break-inducing agents. Therefore, kinetoplastid AP endonuclease proteins warrant further investigation regarding their involvement in drug resistance mechanisms and survival in the host.

2. Materials and methods

2.1. Cloning, expression and purification of Trypanosoma brucei TBAPE1

The *TBAPE1* gene was amplified by PCR from *T. brucei* 449 genomic DNA with the following primers: 5′-AGG GCA ATT AAT ATG CCA CCG AAA AAA CTA TCC-3′ and 5′-GTC ATC GGA TCC TTA CTT ACG GAG CCA CAT CTG -3′, digested with AseI and BamHI and cloned into NdeI and BamHI sites of pET28a vector (Novagen) to generate pET28-TBAPE1. The N287A mutant gene was generated by PCR amplification with primers 5′-C ATA TGG GCT GGA GAC CTT GCT GTA GCG GAG CGC GAT TAT G′ and 5′-GTC ATC GGA TCC TTA CTT ACG GAG CCA CAT CTG -3′ on the plasmid template pET28-TBAPE1. The DNA fragment containing the desired mutation (underlined) was digested with NdeI and BamHI and cloned into pET28-TBAPE1 replacing the corresponding NdeI–BamHI fragment.

Plasmids pET28-TBAPE1 and pET28-N287A were transformed into BL21(DE3) for subsequent purification. Histidine-tagged TBAPE1 proteins were purified on Ni^{2+}-charged HiTrap Chelating HP columns (GE Healthcare) as described [27]. The histidine-tagged protein containing eluates were concentrated and applied to a Superdex-75 gel filtration column (GE Healthcare). The fusion proteins were eluted in phosphate buffer (50 mM sodium phosphate pH 7.0, 150 mM NaCl and 10 mM 2-mercaptoethanol), aliquoted and stored in 20% (v/v) glycerol at −80 °C.

Table 1
Schematic representation of the DNA duplexes used as substrate in this study.

Tetrahydrofuran (F)	5′-C C T GCCCTG **F** GCAGCTGCTGG-3′ 3′-G G A CGGGACACGTCGACGACC-5′
Uracil (U)	5′-C C T GCCCTG **U** GCAGCTGCTGG-3′ 3′-G G A CGGGACGCGTCGACGACC-5′
Phosphoglycolate (3′-PG)	3′-PG P 5′-C C T GCCCTG GCAGCTGCTGG-3′ 3′-G G A CGGGACACGTCGACGACC-5′
Phosphate (3′-P)	3′-P P 5′-C C T GCCCTG GCAGCTGCTGG-3′ 3′-G G A CGGGACACGTCGACGACC-5′

2.2. Enzymatic assays

Oligonucleotide substrates were generated as previously described [27]. A 21-mer oligonucleotide containing a tetrahydrofuranyl residue (F) (Eurogentec) or a single uracil base (U) (Trevigen) was employed as substrate for AP endonuclease and uracil DNA-glycosylase activity assays respectively. The 9-mer oligonucleotides harboring a phosphoglycolate (3′-PG) or a phosphate group (3′-P) at the 3′ terminus and the oligonucleotide with a 5′-phosphate (5′-P) were synthesized by Eurogentec and used to determine 3′-phosphodiesterase and 3′-phosphatase activities. The lesion-containing oligonucleotides were labeled at the 5′-end using [γ-^{32}P]ATP (3000 Ci/mmol; Perkin Elmer) and T4 polynucleotide kinase (New England Biolabs). The ^{32}P-labeled oligonucleotides were purified using MicroSpin G25 columns (GE Healthcare) and annealed to 2-fold molar excess of the complementary strand by incubating at 95 °C for 2 min, followed by slow cooling to room temperature. F and U were annealed to a complementary oligonucleotide containing adenine or guanine opposite the lesion respectively; 3′-PG and 3′-P were annealed to a complementary oligonucleotide and to oligonucleotide 5′-P to produce a single-strand break (Table 1).

In a standard reaction (10 μl final volume), 50 fmol of the corresponding ^{32}P-labeled duplex DNA was incubated in reaction buffer (20 mM Tris–HCl pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 1% (w/v) bovine serum albumin, BSA) with increasing amounts of TBAPE1, N287A or protein from total cell extracts as indicated in the figures. Reactions were carried out at 37 °C for 5 min and stopped by adding 10 μl of formamide dye, followed by heating for 5 min at 95 °C before loading onto the gels. To measure uracil glycosylase activity, the total amount of AP sites generated was determined by incubating the reaction mixture with 5 μl of a solution