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Trypanosomes lacking uracil-DNA glycosylase are hypersensitive to antifolates and present a mutator phenotype

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ABSTRACT

Cells contain low amounts of uracil in DNA which can be the result of dUTP misincorporation during replication or cytosine deamination. Elimination of uracil in the base excision repair pathway yields an abasic site, which is potentially mutagenic unless repaired. The *Trypanosoma brucei* genome presents a single uracil-DNA glycosylase responsible for removal of uracil from DNA. Here we establish that no excision activity is detected on U:G, U:A pairs or single-strand uracil-containing DNA in uracil-DNA glycosylase null mutant cell extracts, indicating the absence of back-up uracil excision activities. While procyclic forms can survive with moderate amounts of uracil in DNA, an analysis of the mutation rate and spectra in mutant cells revealed a hypermutator phenotype where the predominant events were GC to AT transitions and insertions. Defective elimination of uracil via the base excision repair pathway gives rise to hypersensitivity to antifolates and oxidative stress and an increased number of DNA strand breaks, suggesting the activation of alternative DNA repair pathways. Finally, we show that uracil-DNA glycosylase defective cells exhibit reduced infectivity in vivo demonstrating that efficient uracil elimination is important for survival within the mammalian host.

1. Introduction

Trypanosoma brucei is the etiologic agent of both African trypanosomiasis and nagana in humans and animals, respectively. The limited number of efficient drugs, toxicity issues and cases of resistance fully justify the need of new drugs to control the disease. Pyrimidine biosynthesis and the DNA repair pathway are metabolic processes that offer therapeutic potential. Uracil is a base that is not usually present in DNA yet it can appear as a result of cytosine deamination, a spontaneous or enzymatic event, or dUMP misincorporation during DNA replication. Uracil in DNA is mutagenic and cytotoxic and must be eliminated via the base excision repair pathway (BER) (Auerbach et al., 2005). BER is the major route for the repair of base lesions in DNA without helix distortions (Krokan et al., 1997). Uracil-DNA glycosylase (UNG) is the first enzyme of the uracil excision repair pathway, which recognizes and cleaves this promutagenic base off the deoxyribose phosphate DNA backbone (Lindahl et al., 1977). Subsequently, the abasic site is processed by an AP-endonuclease to generate a 5′-deoxyribosephosphate and a 3′-OH end. The repair is completed by short patch repair where a single nucleotide is inserted or by long patch repair in which two to eight nucleotides are inserted prior to ligation (Dogliotti et al., 2001; Stierum et al., 1999). In *Trypanosoma cruzi* short patch repair appears to be the preferred mechanism for uracil repair (Pena-Diaz et al., 2004).

Whilst high amounts of uracil in DNA are detrimental, this base has a central role in certain biological processes. Thus, U:G mispairs generated by controlled enzymatic deamination are key intermediates in the maturation of antibodies in somatic hypermutation and in the antibodies repertoire production with different effector functions in class switch recombination (Rada et al., 2002; Imai et al., 2003). Additionally, uracil incorporation into retroviral DNA mediated by the host cell is a defense mechanism against retroviral infection (Bishop et al., 2004; Mangeat et al., 2003).

UNG proteins belong to a highly conserved class of repair enzymes which have exquisite specificity for uracil in both single and double-stranded DNA, although they are also capable of removing uracil-derived bases (Krokan et al., 1997). The *T. brucei* UNG belongs to the Family-1 of UDGs, which is found in the majority of the species with certain exceptions such as *Drosophila melanogaster* and Archaea (Aravind and Koonin, 2000).

Two isoforms of uracil-DNA glycosylases exist in human cells, a mitochondrial (UNG1) and a nuclear (UNG2) species, which

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are encoded by the same *UNG* gene but differ in the N-terminal domain that is required for subcellular sorting (Nilsen et al., 1997). On the other hand, yeast contains a single uracil-DNA glycosylase (Ung1) with a dual localization in both mitochondrion and nucleus (Chatterjee and Singh, 2001). In humans at least three different DNA glycosylases, in addition to UNG, may remove uracil from DNA; SMUG1, TDG and MBD4 (Sousa et al., 2007), while in *T. brucei* these three latter enzymes are absent from the genome.

UNG is not essential for cell proliferation in several organisms, however numerous defects arise as a consequence of the absence of activity. The lack of UNG results in the development of B-cell lymphomas in mice (Nilsen et al., 2003; Andersen et al., 2005) and an increase in the spontaneous mutation frequencies of the nuclear and mitochondrial genomes in yeast and mouse cells (Nilsen et al., 2000; An et al., 2005). UNG inhibition by small interfering RNA-directed knockdown reduced cell proliferation, induced apoptosis, and increased cellular sensitivity to genotoxic stress in human prostate cancer cells (Pulukuri et al., 2009). Higher sensitivity to oxidative stress has been described in the brain of UNG deficient mice (Endres et al., 2004), and in *Arabidopsis thaliana* the absence of UNG activity increases the resistance to compounds that enhance dUMP incorporation into DNA such as 5-fluorouracil (5-FU) (Cordoba-Canero et al., 2010). Finally, Akbary and collaborators have established a relationship between mitochondrial UNG and defense against oxidative stress, since the mRNA levels of UNG1 but not of UNG2 were up-regulated after exposure to hydrogen peroxide (Akbari et al., 2007).

The aim of the present study was to analyze the genomic defects arising as a consequence of defective uracil elimination in *T. brucei*. We show that while considerable levels of uracil are tolerated in the DNA of trypanosomes, increased mutation rates and DNA fragmentation result as a consequence of UNG deficiency. The lack in uracil glycosylase renders cells hypersensitive to antifolates and hydrogen peroxide with reduced infectivity in vivo while overexpression of the enzyme is detrimental to procyclic trypanosomes which exhibit cell cycle alterations and severe impairment of growth. These observations underline the importance of the TbUNG enzyme in the preservation of genomic integrity and indicate a role for BER in the parasite defense mechanisms that operate within the mammalian host.

2. Materials and methods

2.1. Trypanosome growth and transfection

The *T. brucei* single-marker bloodstream form (BF) (Wirtz et al., 1999) and the procyclic cell line 449 (PF) (Estevez et al., 2001) were used to generate *UNG*-KO and overexpressing UNG (UNG/OE) transfectants. Bloodstream trypanosomes were cultured at 37 °C and 5% CO_2 in HMI-9 with 10% (v/v) fetal bovine serum (FBS) and procyclic forms were cultured at 28 °C in SDM-79 supplemented with 10% FBS and 7.5 μg ml^{-1} Haemin.

Transfections of bloodstream and procyclic parasites using pGRV20, pGRV21, pGRV23b, pGRV33 and pGR11 linearized with NotI were carried out as previously described (Wirtz et al., 1998, 1999) and concentrations of selecting drugs were 5 μg ml^{-1} and 10 μg ml^{-1} of blasticidin (pGRV20), 5 μg ml^{-1} and 50 μg ml^{-1} of hygromycin (pGRV21) and 0.1 μg ml^{-1} and 1 μg ml^{-1} of puromycin (pGRV23b and pGRV33) for bloodstream and procyclics forms, respectively.

T. brucei parental and *UNG*-KO procyclic cell lines were transfected with linearized pGR11 plasmid containing the Herpes simplex type 1 virus thymidine kinase gene (kindly provided by Antonio M. Estevez), and the resultant *T. brucei* PF TK and *T. brucei* PF *UNG*-KO/TK cell lines were selected with 1 μg ml^{-1} of puromycin. The resulting strains were used to study frequency and spectra of mutations.

2.2. Generation of a T. brucei UNG knockout cell line

To generate the *T. brucei* gene replacement cassettes, a 5′-UTR fragment of 432 bp (position 50–482) and a 3′-UTR fragment of 287 bp (position 20–307) of the regions flanking the open reading frame of *T. brucei UNG* gene were amplified by PCR from wild type *T. brucei* 427 genomic DNA. The primers used were designed from the sequence found in the Gene DB database (Tb10.61.2910), which were 5′-CGC GGC CGC ACT GGA TTC TCC TGG ATT G-3′ (NotI restriction site underlined) and 5′-GCC TCG AGG GGA AAA AGC TAG TCC CTC TG-3′ (XhoI) in the case of 5′-UTR region, and 5′-GCA GGC CTT TTG TTG AGG GAC TAT AC-3′ (StuI) and 5′-GGC TAG CGC GGC CGC GTG ACA AAA GAC GTG AAC CCC CGG-3′ (NheI and NotI) for 3′-UTR region. Firstly, to remove one of the two *TbUNG* alleles, both 5′-UTR and 3′-UTR regions of *UNG* gene were cloned in the pHD887 plasmid, kindly provided by Christine Clayton (ZMBH, Heidelberg, Germany), flanking the blasticidin S transferase (*BSD*) resistance gene as a selectable marker yielding the construct pGRV20. To remove the second *TbUNG* allele, the hygromycin phosphotransferase (*HYG*) selectable marker obtained from pGR19 (Clayton et al., 2005) by digestion with SnaBI was subcloned into SnaBI-digested pGRV20 replacing the *BSD* resistance gene and yielding the construct pGRV21.

2.3. Generation of the T. brucei UNG/OE and T. brucei UNG-c-myc/OE cell lines

The cell lines that overexpress the UNG protein were obtained by introduction of a *TbUNG* ectopic copy in both parental and *UNG*-KO cell lines in both bloodstream and procyclic forms. Wild type *T. brucei* 427 genomic DNA was used as template for amplification of the *UNG* coding sequence by PCR using specific primers designed from the sequence found in the GeneDB database (Tb10.61.2910), which were 5′- GCC ATA TGG TGC AAC GGA CAC TGT TTG AC-3′ (NdeI restriction site underlined) and 5′- GCG GAT CCC TAC TTT CCC TTA CTG AGG C -3′ (BamHI restriction site underlined). The amplified fragment was cloned in an expression plasmid, which affords regulated expression of the corresponding gene from a procyclin promoter responsive to tetracycline and a *PAC* resistance gene as selectable drug. This plasmid was created from pGR11 by introduction of a tetracycline-inducible *parp* promoter and rDNA promoter to transcribe the *UNG* gene and *PAC* resistance genes respectively. The resultant plasmid was named pGRV23b.

A second construction for overexpressing the UNG with a C-terminal fusion of the c-myc epitope was made from pGRV23b plasmid. First, the *UNG* gene in pGRV23b plasmid was substituted by a *UNG* gene that lacks the stop codon, obtained by PCR amplification with the following oligonucleotides: 5′-GCC ATA TGG TGC AAC GGA CAC TGT TTG AC-3′ (NdeI restriction site underlined) and 5′-GCG GAT CCG TTA ACC TTT CCC TTA CTG AGG CAT AC-3′ (BamHI and HpaI restriction sites underlined). The sequence coding for the c-myc epitope tag was inserted as a linker into the HpaI and BamHI sites yielding plasmid pGRV33. The linker was made by annealing sense (5′-AAC GAG GAG CAG AAG CTG ATC TCA GAG GAG GAC CTG TAG-3′) and antisense (5′-CTA CAG GTC CTC CTC TGA GAT CAG CTT CTG CTC CTC GTT-3′) oligonucleotides. The amino acid sequence for the carboxy-terminal tag is SKGKVNEEQKLISEEDL* (c-myc sequence is underlined, asterisk indicates stop codon).