DNA Repair 13 (2014) 32–41

Contents lists available at ScienceDirect

DNA Repair

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

The contribution of Nth and Nei DNA glycosylases to mutagenesis in *Mycobacterium smegmatis*

Nabiela Moolla, Vivianne J. Goosens [1], Bavesh D. Kana, Bhavna G. Gordhan *

DST/NRF Centre of Excellence for Biomedical TB Research, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand and the National Health Laboratory Service, P.O. Box 1038, Johannesburg 2000, South Africa

ARTICLE INFO

Article history:
Received 9 September 2013
Received in revised form
21 November 2013
Accepted 26 November 2013
Available online 15 December 2013

Keywords:
Mycobacterium tuberculosis
Base excision repair
Formamidopyrimidine DNA glycosylase
EndonucleaseVIII
Endonuclease III
Mutagenesis
DNA repair

ABSTRACT

The increased prevalence of drug resistant strains of *Mycobacterium tuberculosis* (Mtb) indicates that significant mutagenesis occurs during tuberculosis disease in humans. DNA damage by host-derived reactive oxygen/nitrogen species is hypothesized to be critical for the mutagenic process in Mtb thus, highlighting an important role for DNA repair enzymes in maintenance of genome fidelity. Formamidopyrimidine (Fpg/MutM/Fapy) and EndonucleaseVIII (Nei) constitute the Fpg/Nei family of DNA glycosylases and together with EndonucleaseIII (Nth) are central to the base excision repair pathway in bacteria. In this study we assess the contribution of Nei and Nth DNA repair enzymes in *Mycobacterium smegmatis* (Msm), which retains a single *nth* homologue and duplications of the Fpg (*fpg1* and *fpg2*) and Nei (*nei1* and *nei2*) homologues. Using an *Escherichia coli nth* deletion mutant, we confirm the functionality of the mycobacterial *nth* gene in the base excision repair pathway. Msm mutants lacking *nei1*, *nei2* and *nth* individually or in combination did not display aberrant growth in broth culture. Deletion of *nth* individually results in increased UV-induced mutagenesis and combinatorial deletion with the *nei* homologues results in reduced survival under oxidative stress conditions and an increase in spontaneous mutagenesis to rifampicin. Deletion of *nth* together with the *fpg* homolgues did not result in any growth/survival defects or changes in mutation rate. Furthermore, no differential emergence of the common rifampicin resistance conferring genotypes were noted. Collectively, these data confirm a role for Nth in base excision repair in mycobacteria and further highlight a novel interplay between the Nth and Nei homologues in spontaneous mutagenesis.

1. Introduction

Upon infection, *Mycobacterium tuberculosis* (Mtb) is engulfed by host alveolar macrophages where it is exposed to reactive oxygen intermediates (ROI) and nitrogen intermediates (RNI) generated as part of the host's immune response to contain infection [1]. It has been hypothesized that oxidative stress imposed by the host results in damage of mycobacterial DNA through the formation of abasic sites, single and double stranded breaks, and in base damage through oxidative deamination of cytosine or conversion of guanine to 7,8-dihydro-8-oxoguanine (8-oxoG). Consistent with this, *Mtb* encodes numerous enzymes involved in the base excision repair (BER) and nucleotide excision repair (NER) pathways [2–4], some of which have been shown to be required for survival in animal models of infection [5–8].

In the first step of the BER pathway, cytotoxic and mutagenic DNA bases are recognized and excised by specialized DNA glycosylases, resulting in the formation of an apurinic/apyrimidine (AP) site which is further processed by downstream components of the pathway [9]. Oxidation of guanine can create both cytotoxic and pro-mutagenic lesions – 2,6-diamino-5-formamidopyrimidine (FapyG) is an example of a cyotoxic (replication-blocking) lesion – whereas 8-oxoguanine (8-oxoG) is pro-mutagenic by virtue of its strong miscoding properties. During replication, unrepaired 8-oxoG can mispair with adenine leading to G → T transversions [10]. 8-oxoG can be further oxidized to form other pro-mutagenic lesions such as spiroiminodihydrantoin (Sp) and 5-guanidinohydantoin (Gh) which cause G → T and G → C transversions, respectively [11].

DNA glycosylases that remove oxidized DNA base pairs fall into two families based on structural and sequence homology. The Fpg/Nei family consists of structurally related enzymes including formamidopyrimidine DNA glycosylases (Fpg/MutM/Fapy) which recognize and excise oxidized purines such as 8-oxoG, FapyG and 4,6-diamino-5-formamidopyrimidine (FapyA), and endonuclease

* Corresponding author. Tel.: +27 11 4899135; fax: +27 11 4899397.
E-mail address: bhavna.gordhan@nhls.ac.za (B.G. Gordhan).
[1] Present address: Department of Medical Microbiology, University of Groningen, University Medical Center Groningen, Hanzeplein 1, P.O. Box 30001, 9700 RB Groningen, The Netherlands.

1568-7864/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.dnarep.2013.11.003

VIII (Nei), which repair oxidatively damaged pyrimidines and FapyA [12–15]. The Nth superfamily, represented by endonuclease III (Nth), shares some substrate specificity with Nei [12] and is primarily responsible for removing oxidized pyrimidines [16–23]. In organisms such as *Mycobacterium leprae*, *Haemophilus influenza*, and *Helicobacter pylori* there is preferential conservation of Nth over Nei [24–27], in contrast other bacteria have multiple copies of the *nth* homologue with deletion of any one having extreme consequences [28–32]. Furthermore, transcriptional analysis using inhibitors of Mtb metabolism revealed up-regulation of *nth* implicating an integral role of this enzyme in DNA turnover [33].

In mycobacteria, there is a single homologue for *nth* and a duplication of the Fpg/Nei homologues in both *Mycobacterium smegmatis* and Mtb encoding the necessary domains for Fpg and Nei catalytic function, Fig. 1 [2,22,24,34,35]. However, *M. leprae*, an organism that has undergone massive genome decay [36,37], retains only one Fpg/Nei homologue [34], implying that the expanded complement of genes in the other pathogenic mycobacteria may be a deliberate adaptation to survive more varied environmental conditions.

Recent studies demonstrate overlapping but also altered substrate preferences for the Mtb enzymes [22,35,38]. In this regard, Nth from Mtb was shown to recognize a variety of oxidized bases, and both Nei1 and Nth excise thymine glycol [22]. *M. smegmatis* Fpg1 was shown to recognise and excise 8-oxoG and Fapy lesions [38] and similarly, Mtb Fpg1 was shown to be a functionally active enzyme that primarily targets oxidised purines [34]. In accordance with previous predictions [2], Mtb Fpg2 has neither DNA binding nor glycosylase/lyase activity, consistent with the absence of the highly conserved N-terminal domain [22,34] whilst the *M. smegmatis* Fpg2 has maintained the necessary domains for Fpg protein function with strong resemblances to the Fpg1 homologue and to previously characterised Fpg family of DNA glycosylases [15]. Although Mtb Nei1 showed specificity for oxidized pyrimidines as well as uracil DNA glycosylase activity, Nei2 displayed no activity *in vitro* [22]. In addition to confirming functional differentiation within the Fpg/Nei family, these findings suggest that Mtb sustains significant oxidative DNA damage during infection [39] and BER defects resulting from loss of specific DNA glycosylase activity may reduce the replicative fitness of Mtb *in vivo* [40].

In this study, we investigate the individual and collective role of the Fpg/Nei family and Nth superfamily of DNA glycosylases in mycobacterial growth, survival and mutation avoidance. We demonstrate that the Fpg/Nei homologues or Nth can be individually eliminated in *M. smegmatis* without significant phenotypic consequences for growth, survival and mutagenesis. However, deletion of *nth* results in increased UV-induced mutagenesis whilst combinatorial deletion with the *nei* homologues leads to decreased survival under oxidative stress conditions and increased spontaneous mutagenesis.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are detailed in Table S1. *Escherichia coli* strains were grown in Luria–Bertani broth (LB) or on Luria agar (LA). *M. smegmatis* strains were grown in Middlebrook 7H9 media (Difco) supplemented with 0.2% glycerol, 0.2% glucose, 0.085% NaCl and 0.05% Tween 80 (referred to as 7H9) or Middlebrook 7H10 media (Difco) supplemented with 0.5% glycerol, 0.2% glucose and 0.085% NaCl (referred to as 7H10). Where appropriate, the media for *E. coli* cultures was supplemented with ampicillin (Amp) and kanamycin (Km) at final concentrations of 100 and 50 $\mu g\,ml^{-1}$, respectively and for *M. smegmatis* cultures with Km at a final concentration of 20 $\mu g\,ml^{-1}$.

2.2. Allelic exchange mutagenesis

Allelic exchange mutagenesis was carried out using previously described methods [41,42] using the knockout constructs pΔ*fpg1*, pΔ*fpg2*, pΔ*nei1*, pΔ*nei2* and pΔ*nth* which carried deletions in the *fpg1*, *fpg2*, *nei1*, *nei2* and *nth* genes respectively (Tables S1 and S2). All mutants were screened by PCR amplification at all three loci using the primers designed to differentiate the mutant and wild type alleles (Table S3). Mutant genotypes were further confirmed by Southern blot hybridization (Fig. S3).

2.3. Generation of *E. coli* and *M. smegmatis* nth complemented strains

The DNA encoding the *nth* gene from *M. smegmatis* was amplified by PCR using primers outlined in Table S3, followed by blunt end cloning into the *Ecl136*II restriction site of the integrating vector, pTWEETY. The resulting vector was electroporated into both the *E. coli* (EcΔ*nth*) and *M. smegmatis* (Δ*nth*, Δ*fpg1* Δ*fpg2* Δ*nth*, Δ*nei2* Δ*nei1* Δ*nth*) *nth* deficient mutant strains to generate the respective genetically complemented strains. Integration of the vector was confirmed by PCR using the primers listed in Table S2. In addition, expression of *nth* in *M. smegmatis* was measured by reverse transcriptase-polymerase chain reaction (RT-PCR) using the primers listed in Table S4.

2.4. Gene expression analysis by RT-PCR

To monitor the expression of *nth* in the mutant strains and complemented derivatives, RNA was extracted from mid-logarithmic phase cultures (OD_{600nm} = 0.35), using previously described methods [43]. RT-PCR analysis of *nth* and *sigA* gene expression in the wild-type and mutant strains was performed using the primer sets described in Table S4. Synthesis of cDNA was carried out at 65 °C for 5 min using 2 μg of RNA in a 20 μl reaction mixture containing 1 × RT buffer, 25 mM magnesium chloride, 0.1 M dithiothreitol [DTT] and 200 U/μl SuperScript^{TM} III RT (Invitrogen). For RT-PCR, 2 μl of cDNA or 10-fold serial dilutions of *M. smegmatis* genomic DNA (standard curve) was used for amplification in an Eppendorf MasterCycler with Faststart Taq DNA Polymerase using primers listed in Table S4 and following manufacturer's instructions. Products were visualized on a 2% agarose gel (Fig. S4).

2.5. Rifampicin mutation frequency in *E. coli*

E. coli cultures were grown in LB to OD_{600nm} = 0.7 and 100 μl of each culture was spread onto LA containing rifampicin (100 $\mu g\,ml^{-1}$) to determine the number of spontaneous rifampicin resistant (rif^R) mutants. Each of the cultures was serially diluted and 100 μl of the appropriate dilution was plated in duplicate on LA media to determine cell numbers. The frequency was calculated as the ratio of rif^R mutants to the total number of cells.

2.6. Growth of mutant strains in vitro

To assess growth of the mutant strains in axenic culture, 25 ml cultures were grown in 7H9 broth in 250 ml culture flasks with shaking for 36 h. Growth was monitored by enumerating colony forming units (CFUs) on 7H10 over 24–48 h.

2.7. Survival and mutagenesis under oxidative stress

M. smegmatis cultures were grown in Middlebrook 7H9 media (Difco) supplemented with 0.2% glycerol, 0.2% glucose, 0.085% NaCl and 0.05% Tween 80 to OD_{600nm} = 0.35 and treated with 2.5 mM H_2O_2. Cell viability was monitored at 2 h intervals over a 6 h period