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Brief report

3-Methyladenine DNA glycosylase is important for cellular resistance to psoralen interstrand cross-links

*Ayelet Maor-Shoshani, Lisiane B. Meira, Xuemei Yang, Leona D. Samson**

Biological Engineering Department and Center for Environmental Health Sciences, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139, United States

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ABSTRACT

DNA interstrand cross-links (ICLs), widely used in chemotherapy, are cytotoxic lesions because they block replication and transcription. Repair of ICLs involves proteins from different repair pathways however the precise mechanism is still not completely understood. Here, we report that the 3-methyladenine DNA glycosylase (Aag), an enzyme that initiates base excision repair at a variety of alkylated bases, is also involved in the repair of ICLs. *Aag*$^{-/-}$ mouse embryonic stem cells were shown to be more sensitive to the cross-linking agent 4,5′,8-trimethylpsoralen than wild-type cells, but no more sensitive than wild-type to the psoralen derivative Angelicin that forms only monoadducts. We show that γ-H2AX foci formation, a marker for double strand breaks that are formed during ICL repair, is impaired in psoralen treated *Aag*$^{-/-}$ cells in both quantity and kinetics. However, in our *in vitro* system, purified human AAG can neither bind to the ICL nor cleave it. Taken together, our results suggest that Aag is important for the resistance of mouse ES cells to psoralen-induced ICLs.

1. Introduction

Cells are constantly exposed to DNA damaging agents. To overcome this constant assault, many different pathways have evolved to repair the damage thus restoring normal replication and transcription. Approximately 150 genes participate in different pathways of damage repair or tolerance in humans [1]. For every type of DNA damage, there is at least one repair mechanism or pathway, and some kinds of damage can be acted upon by several different pathways.

The enzyme 3-methyladenine DNA glycosylase (AAG in human, Aag in mouse) is specialized in removing various types of modified bases from the DNA, such as 3-methyladenine, 7-methylguanine, hypoxanthine (Hx) and 1,N^6-ethenoadenine, among others [2,3]. AAG recognizes the damaged base and initiates the base excision repair (BER) process by cleaving the *N*-glycosylic bond between the damaged base and the deoxyribose, creating an abasic site [2,4]. In its simplest form, BER is completed by the action of AP endonuclease (APE1 in human) which cleaves at the abasic site, DNA polymerase β which trims the 5′ end and fills in the missing nucleotide, and DNA ligase which seals the nick [5–7].

Mouse embryonic stem (ES) cells that lack Aag are more sensitive than wild-type to alkylating agents such as methyl methanesulfonate (MMS) [8,9]. Interestingly, it was shown that *Aag*$^{-/-}$ mouse ES cells are also sensitive to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and mitomycin C (MMC), both of which are chemotherapeutic agents known to induce

* *Corresponding author*. Tel.: +1 617 258 7813; fax: +1 617 253 8099.
E-mail address: lsamson@mit.edu (L.D. Samson).
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DNA interstrand cross-links (ICLs) [8,10]; both BCNU and MMC initially induce monoadducts, only some of which can further react to form ICLs. Although Aag had no apparent *in vitro* glycosylase activity on double stranded DNA containing a MMC ICL or N^2-guanine monoadduct [10], the sensitivity of $Aag^{-/-}$ cells to MMC could be explained by a possible role in the repair of yet another *in vivo* monoadduct formed by MMC. As for BCNU, it produces lesions at both the N7 and the O^6 positions of guanine. O^6-Chloroethylguanine is normally repaired via direct reversal by the O^6-methylguanine DNA methyltransferase (MGMT). However, when O^6-chloroethylguanine escapes repair by MGMT it can go on to rearrange into an 1,O^6-ethanoguanine lesion, which in turn goes on to react with the cytosine opposite, forming an ICL. 1,O^6-Ethanoguanine is structurally similar to 1,N^6-ethenoadenine that is a known substrate for Aag. Therefore, Aag might protect ES cells against BCNU and MMC by repairing monoadducts that have the potential to form ICLs, rather than by repairing ICLs *per se* [10]. In addition, mutations in the yeast *MAG1* gene, the functional homologue of *Aag*, render cells sensitive to nitrogen mustard treatment [11].

ICLs are very detrimental lesions to the cell, since they block fundamental processes required for cell survival—namely replication and transcription. The mechanisms for repair of ICLs in bacteria and yeast are somewhat understood, and appear to involve the nucleotide excision repair (NER) and homologous recombination (HR) pathways, as well as translesion synthesis (TLS) [12]. Likewise, ICL repair in mammalian cells is believed to involve some proteins from NER, HR and TLS pathways, along with other proteins [12–14]. The major repair pathway is believed to be both replication and recombination dependent, although two other minor repair pathways have been proposed [15–17]. According to most models, the major repair pathway for ICLs in mammals is initiated when the replication fork is stalled at the lesion, followed by strand cleavage on the fork side of the ICL, generating a collapsed replication fork with a one-sided double strand break (DSB) [13,14,18–21]. This cleavage is thought to be mediated by a structure-specific endonuclease, either Mus81-Eme1 [22] or XPF-ERCC1 [23]. Thereafter, XPF-ERCC1 cleaves the DNA on the other side of the cross-link, unhooking it from the dsDNA [14,23]. The requirement for only XPF-ERCC1 from the NER machinery for that step explains the hypersensitivity of *XPF* and *ERCC1* mutants to ICLs agents, while other NER mutants exhibit only mild sensitivity [13,24]. After the lesion is unhooked and thus tethered to only one strand, the gap opposite can be filled via lesion bypass by a translesion polymerase. Once the gap opposite the ICL is filled, a simple NER process can excise the unhooked lesion and the gap will be filled by a polymerase, restoring the continuity of the DNA. The one sided DSB that was formed at the replication fork at the beginning of the process then needs to participate in replication fork restoration, probably by the action of the homologous recombination machinery. Strong evidence supports the involvement of homologous recombination in ICL repair, since mutations in the *XRCC2*, *XRCC3*, *RAD51C*, and *RAD51D* genes result in severe sensitivity to ICL inducing agents [13,25–27].

Additional proteins from other repair pathways have been shown to be involved in ICL repair. hMutSβ appears to be required for the recognition and uncoupling of psoralen ICLs *in vitro* [28]. Moreover, MMR deficient cells (MSH2 mutants) are hypersensitive to psoralen ICLs, but do not have lower frequencies of cross-link-induced mutations, suggesting that MMR may be involved in a relatively error-free mechanism to process ICLs [29]. The Fanconi Anemia proteins are thought to have a role both in the regulation of ICL repair, and in the actual repair reaction through FANCM [30,31] and FANCJ [11,32,33] (reviewed in [18] and [34]). BRCA2, which plays a role in homologous recombination [35] is the Fanconi Anemia gene FANCD1 [36]. Using an *in vitro* assay it was shown that BRAC2 participates in the repair of DSBs generated when replication forks encounter ICLs [37]. Monoubiquitination of FANCD2 promotes BRCA2 loading into chromatin complexes, that are required for normal homology-directed DNA repair [38,39]. In addition, it was suggested that the pre-mRNA spliceosome complex Pso4 together with the Werner helicase are required for ICL processing, in coordination with BRAC1 [40,41].

Here, we show that $Aag^{-/-}$ mouse ES cells are more sensitive than wild-type cells to the interstrand cross-linking agent 4,5′,8-trimethylpsoralen (TMP), while they have similar sensitivity to Angelicin, a psoralen derivative which forms mainly monoadducts and no cross-links. The formation of γ-H2AX foci, markers for DSBs, was delayed and less robust in $Aag^{-/-}$ cells compared to wild-type cells following treatment with TMP, while there was no difference following treatment with Angelicin. Caspase-3 activation, a marker for apoptosis, was higher in $Aag^{-/-}$ than in wild-type cells following the cross-linking treatment. Taken together, our results suggest an important role for Aag in cellular resistance to psoralen ICLs.

2. Material and methods

2.1. Reagents and proteins

Cells were treated with either MMS, 4,5′,8-trimethylpsoralen or Angelicin (Sigma–Aldrich). TMP and Angelicin treatments were followed by UVA irradiation using a UVA lamp at 365 nm (UVP, CA). Monoclonal anti-phospho-Histone H2AX antibody was from Upstate Technology (NY). Alexa fluor 594 goat anti mouse, Alexa fluor 647 goat anti rabbit, and ProLong Gold with DAPI were from Invitrogen. Rabbit anti-active Caspase-3 was from BD Pharmingen. Giemsa staining solution (0.02%, w/v), 10× TBS, Triton-X were from Sigma. 16% solution EM grade paraformaldehyde was from Electron Microscopy Sciences. 10% Twin 20 and blotting grade blocker non-fat dry milk were from Bio-Rad.

Cell culture reagents: DMEM, L-glutamine, Penicillin–Streptomycin, 2-mercaptoethanol, Trypsin–EDTA were from Invitrogen, Fetal Bovine Serum (FBS) was from Hyclone.

2.2. Cells

AB1 ES cells and their $Aag^{-/-}$ derivative [8] were cultured on SNL76/7 feeder cells that were mitotically inactivated by gamma irradiation, in DMEM, supplemented with 15% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine,