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Substrate binding pocket residues of human alkyladenine-DNA glycosylase critical for methylating agent survival

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ABSTRACT

Human alkyladenine-DNA glycosylase (AAG) initiates base excision repair (BER) of alkylated and deaminated bases in DNA. Here, we assessed the mutability of the AAG substrate binding pocket, and the essentiality of individual binding pocket amino acids for survival of methylation damage. We used oligonucleotide-directed mutagenesis to randomize 19 amino acids, 8 of which interact with substrate bases, and created more than 4.5 million variants. We expressed the mutant AAGs in repair-deficient *Escherichia coli* and selected for protection against the cytotoxicity of either methylmethane sulfonate (MMS) or methyl-lexitropsin (Me-lex), an agent that produces 3-methyladenine as the predominant base lesion. Sequence analysis of 116 methylation-resistant mutants revealed no substitutions for highly conserved Tyr^{127} and His^{136}. In contrast, one mutation, L180F, was greatly enriched in both the MMS- and Me-lex-resistant libraries. Expression of the L180F single mutant conferred 4.4-fold enhanced survival at the high dose of MMS used for selection. The homogeneous L180F mutant enzyme exhibited 2.2-fold reduced excision of 3-methyladenine and 7.3-fold reduced excision of 7-methylguanine from methylated calf thymus DNA. Decreased excision of methylated bases by the mutant glycosylase could promote survival at high MMS concentrations, where the capacity of downstream enzymes to process toxic BER intermediates may be saturated. The mutant also displayed 6.6- and 3.0-fold reduced excision of $1,N^6$-ethenoadenine and hypoxanthine from oligonucleotide substrates, respectively, and a 1.7-fold increase in binding to abasic site-containing DNA. Our work provides *in vivo* evidence for the substrate binding mechanism deduced from crystal structures, illuminates the function of Leu^{180} in wild-type human AAG, and is consistent with a role for balanced expression of BER enzymes in damage survival.

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1. Introduction

Alkylating agents are widely dispersed in the environment, occur endogenously, and are frequently used in the treatment of cancer [1,2]. If not repaired, alkylated bases in DNA can be mutagenic and cytotoxic [3]. For example, modification at the N^3 position of adenine can disrupt interaction of DNA polymerases with the template DNA strand [4,5], apparently leading to arrest of the replication machinery and cell death [6–8]. Base excision repair (BER) is a primary mechanism by which cells remove damaged bases from the genome, including alkylated bases, and has been conserved in evolution. BER pathways are initiated by a DNA glycosylase that recognizes the adducted base and hydrolyzes the *N*-glycosylic bond linking the damaged base to the sugar-phosphate backbone [9]. The resulting abasic site is then processed by AP endonuclease or Ap lyase, DNA polymerase and DNA ligase activities [10]. Importantly, abasic sites and the downstream interrupted-strand intermediates can lead to double-strand breaks and are therefore potentially cytotoxic. DNA glycosylase action thus commits cells to a pathway in which each step produces a different and possibly lethal form of damaged DNA, until repair is complete. As a consequence, the balanced expression [11,12] and coordinated action [10] of enzymes in BER pathways are important for survival of DNA damage.

Alkyladenine DNA glycosylases are found in organisms ranging from bacteria and plants to humans [2,13]. The alkyladenine-DNA glycosylase AAG (also known as *N*-methylpurine DNA glycosylase (MPG), or ANPG) is the only known human DNA glycosylase that removes alkylated bases [1,9]. Human AAG is active on a broad range of chemically and structurally diverse substrates. In addition to 3-methyladenine (3-meA), AAG also excises the alkylated purines 7-methylguanine (7-meG) and 3-methylguanine (3-meG) [13]; the oxidative purine deamination products hypoxanthine, xanthine, and oxanine [14,15]; the lipid peroxidation-derived 1,N^6-ethenoadenine (εA) and 1,N^2-ethenoguanine (εG); the oxidative lesion 8-hydroxyguanine [16]; and, very inefficiently, normal guanine [13,17–19]. AAG has been reported to excise hypoxanthine most efficiently, followed by εA and 3-meA, leading to the suggestion that AAG may have primarily evolved as a hypoxanthine-DNA glycosylase [20,21].

Crystallographic studies and molecular modeling [22,23], together with site-specific mutagenesis [24], have provided substantial information concerning the mechanism of action of human AAG and the structural basis for binding adducted purines. Co-crystal structures of AAG complexed with lesion-containing DNA reveal that AAG, like other DNA glycosylases, uses a nucleotide flipping mechanism to recognize target bases in an extrahelical conformation [22,23]. AAG binds in the minor groove of DNA, and a single tyrosine (Tyr^{165}) inserts into the minor groove, intercalating between the bases adjacent to, and occupying the space left by, the flipped-out nucleotide. The flipped-out nucleotide is captured in a substrate binding pocket, and, in the case of εA, stacks between the side chains of Tyr^{127} on one side and His^{136} and Tyr^{159} on the other [23]. Cleavage of the glycosylic bond is catalyzed by a nucleophilic water molecule that is activated by the essential Glu^{125} [20,23]. Many evolutionarily conserved amino acids form the substrate binding pocket [22,24], and site-directed mutagenesis has begun to illuminate their functions. For example, Asn^{169} has been proposed to play an important role in substrate specificity. When a normal guanine is modeled into the substrate binding pocket, the exocyclic amino group clashes with the Asn^{169} side chain [23]; in accord, replacements for Asn^{169} can enhance excision of guanine from mispairs [25]. Other substrate binding pocket residues include Leu^{180}, which lies adjacent to the N^3 position of εA, and is expected to pack closely with the methyl adduct of 3-meA [22].

Our present understanding of the roles of individual substrate binding pocket residues in substrate discrimination by AAG, and their importance for damage survival, is incomplete. Here, we used oligonucleotide-directed random mutagenesis (e.g., 26) and selection for methylating agent survival to examine the replaceability of amino acids that line the AAG substrate binding pocket. Analysis of methylation-resistant mutants identified mutable and immutable amino acids, yielding *in vivo* evidence for an essential role of Tyr^{127} and His^{136} in survival of methylation damage. Our results provide functional validation of crystallographically observed interactions, suggest functions of Leu^{180} in substrate discrimination by wild-type human AAG, and provide a presumptive example of the importance for damage survival of coordinated processing of intermediates in BER pathways.

2. Materials and methods

2.1. Strains and materials

The alkylation repair-deficient strain *E. coli* MV1932 (alkA1 tag) is a derivative of strain AB1157 (see [12]). *E. coli* BL21(DE3) was purchased from Agilent technologies (Santa Clara, CA). Enzymes were from NEB (Ipswich, MA), and oligonucleotides were from IDT (Coralville, IA). *E. coli* endonuclease IV was kindly provided by Dr. S. Bennett (Oregon State University). Anti-AAG (MPG) antibody was obtained from Imgenex (San Diego, CA).

2.2. Creation of pUC-AAG-SK and KX mutant libraries

The starting vector encoded a full-length human AAG cDNA in a pUC19-derived plasmid. A schematic diagram of the construction of the pUC-AAG-SK and pUC-AAG-KX random mutant libraries is shown in Supplementary Fig. S1. The library designations SK and KX refer to the SacII and KpnI, and KpnI and XbaI silent restriction sites, respectively, that were introduced in the AAG coding sequence to allow substitution with oligonucleotide cassettes containing randomized codons. The SK library encodes nine randomized amino acids (Ala^{126}, Tyr^{127}, Leu^{128}, Gly^{129}; Glu^{133}, Ala^{134}, Ala^{135}, His^{136}, and Ser^{137}) and the KX library 10 randomized amino acids (Val^{158}, Tyr^{159}, Ile^{160}; Cys^{167}, Met^{168}, Asn^{169}, Ile^{170}; Val^{179}, Leu^{180}, and Leu^{181}). To permit library construction, the pre-existing SacII and XbaI sites in the pUC19 multiple cloning region were eliminated via restriction digestion, followed by mung bean exonuclease digestion to remove non-complementary termini, filling with Klenow fragment, and religation. To generate silent restric-