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Alkyladenine DNA glycosylase (Aag) in somatic hypermutation and class switch recombination

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ABSTRACT

Somatic hypermutation (SHM) and class switch recombination (CSR) of immunoglobulin (*Ig*) genes require the cytosine deaminase AID, which deaminates cytosine to uracil in *Ig* gene DNA. Paradoxically, proteins involved normally in error-free base excision repair and mismatch repair, seem to be co-opted to facilitate SHM and CSR, by recruiting error-prone translesion polymerases to DNA sequences containing deoxy-uracils created by AID. Major evidence supports at least one mechanism whereby the uracil glycosylase Ung removes AID-generated uracils creating abasic sites which may be used either as uninformative templates for DNA synthesis, or processed to nicks and gaps that prime error-prone DNA synthesis. We investigated the possibility that deamination at adenines also initiates SHM. Adenosine deamination would generate hypoxanthine (Hx), a substrate for the alkyladenine DNA glycosylase (Aag). Aag would generate abasic sites which then are subject to error-prone repair as above for AID-deaminated cytosine processed by Ung. If the action of an adenosine deaminase followed by Aag were responsible for significant numbers of mutations at A, we would find a preponderance of A:T > G:C transition mutations during SHM in an Aag deleted background. However, this was not observed and we found that the frequencies of SHM and CSR were not significantly altered in $Aag^{-/-}$ mice. Paradoxically, we found that Aag is expressed in B lymphocytes undergoing SHM and CSR and that its activity is upregulated in activated B cells. Moreover, we did find a statistically significant, albeit low increase of T:A > C:G transition mutations in $Aag^{-/-}$ animals, suggesting that Aag may be involved in creating the SHM A > T bias seen in wild type mice.

1. Introduction

Somatic hypermutation (SHM) in mammals is a process of secondary diversification of immunoglobulin (*Ig*) genes in activated B cells during which point mutations, and occasional insertions and deletions, are introduced into DNA encoding the antibody variable (V) regions. SHM thereby alters the affinity of antibodies for cognate antigens, a hallmark of adaptive immunity. SHM requires AID, the most likely function of which is to deaminate cytosines in the promoter proximal region DNA of *Ig* genes (reviewed in [1]). Deamination of cytosine (C) in DNA creates a uracil (U) base mispaired

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with guanine (G) that, when present in DNA, is a substrate for DNA repair. During SHM, some of the DNA repair systems that would normally faithfully repair such U:G mismatches paradoxically appear to be co-opted to generate mutations. Proteins of two major systems co-opted for SHM are the uracil glycosylase Ung, and the mismatch repair proteins Msh2, Msh6 (which comprise the Msh2/6 heterodimer), Mlh1, Pms2 (reviewed in [1,2]) and Mlh3 [3,4].

Mice and humans deficient for Ung are proficient for SHM, but the pattern of SHM is altered characteristically in that mutations at C and G are mainly transitions, as if uracils left unrepaired serve as a template for DNA replication [5,6]. As a glycosylase, the function of Ung is to remove U from the DNA backbone, leaving an abasic (AP) site that is processed by concerted action of an AP endonuclease (APE1), and a deoxyribophosphodiesterase (dRPase activity of polymerase (pol) β) to produce a single-strand gap [2]. Error-free filling-in of the gap is accomplished by pol β or the high-fidelity polymerase, δ [7]. However, to explain the SHM pattern shift due to Ung-deficiency, AID/Ung-mediated single-strand gaps are likely to be filled-in using one or more translesion polymerases to generate mutations from all four nucleotides. Of the many recently identified translesion polymerases, particularly polymerase η, ι and θ have been implicated in SHM (reviewed by [8]).

Importantly, Ung-deficiency affects the SHM pattern at C (and G), but has less effect on mutations at A (and T). Mutations at A are at most 52% reduced in Ung-deficient mice [9], presumably, because Ung-dependent mutations at A can arise during long-patch base excision repair (BER) in which polymerases β or δ are replaced by translesion polymerases. In the absence of Ung, these mutations in part seem to depend on functional mismatch recognition by Msh2/6, because, although the pattern of mutations at A is not altered, their frequency is decreased from ~50% in wildtype mice to between 26% and as little as 2% of total mutations in both *Msh2*$^{-/-}$ and *Msh6*$^{-/-}$ mice (reviewed in [1,10]). This decrease in frequency suggests that recognition of the U:G mismatch by MutSα (the Msh2/6 heterodimer) initiates a process that ultimately leads to mutations at adenines, perhaps via recruitment of the error-prone pol η [11,12]. Polη deficiency in mice and humans also leads to a SHM pattern characterized by a low frequency of mutations at A [12–16].

Currently available data suggest that mutations at A can be explained by the activities of Ung and mismatch repair, and errors created by translesion DNA polymerases. However, the precise mechanism by which these mutations arise are not yet known and possibly there are unknown factors that are involved in DNA repair during SHM that could influence mutations at A. Like mutations at C, transition mutations at A are predominant in SHM. A:T → G:C transitions could result from deamination of A to hypoxanthine (Hx), which codes as a G during DNA synthesis [17,18]. Adenosine deaminases exist, although currently are known to act only on RNA [19]. Yet, by analogy to AID, we questioned whether a DNA adenosine deaminase might be involved in SHM. The major DNA repair enzyme for Hx is alkyladenine DNA glycosylase (Aag) [20]. In order to determine whether adenosine deamination plays a role during SHM we examined the SHM pattern in *Aag*$^{-/-}$ mice. We report here that, while the mutational pattern at A is not changed in the *Aag*-deficient animal, activation of B cells leads to a significant induction of Hx glycosylase activity in wild type mice. Furthermore, we see a small yet significant bias towards T:A → C:G transition mutations in the absence of Aag leading us to suggest that Aag glycosylase activity plays a role during SHM.

2. Materials and methods

2.1. *Aag*$^{-/-}$ mice

The generation of the *Aag*$^{-/-}$ mice was previously described [20]. *Aag*$^{-/-}$ animals were backcrossed to a pure C57Bl/6J background (at least 12 backcrosses) and were 6 to 8 months old when received at the University of Chicago. Mice were analyzed soon after arrival, due to quarantine space constraints and institutional animal shipment regulations. Mice were genotyped as described [20]; genomic template DNA for PCR was derived from either PNA-low, sorted B cells from Peyer's patches, or from kidney.

2.2. *Ung*$^{-/-}$ mice

Ung$^{-/-}$ mice were a gift of Barnes and Lindahl [21]. The original C57BL/6J-129SV background mice were maintained in our facility by breeding with C57BL/6J mice. Genotyping of these mice was as described [22].

2.3. *Analysis of SHM in mice*

(i) Cell isolation, staining and flow cytometry.
Peyer's patches were removed, strained, and rinsed twice with cold RPMI culture medium (Invitrogen) prior to staining with antibodies. Cells were stained with anti-mouse B220/CD45-PE (BD Biosciences), anti-mouse PNA-FITC (Sigma-Aldrich), and anti–mouse GL7-FITC (BD Biosciences) antibodies and sorted on a Mo-Flo or FACSAria (BD Biosciences) cell sorter at the Immunology Core Facility at the University of Chicago. PNA-low GL7-low B220+ (non-germinal center B cells) and PNA-high GL7+ B220+ (germinal center, mutating B cells) cells were collected for DNA extraction using DNeasy columns (QIAGEN). Mutations were analyzed in PNA-high B cells of Peyer's patches. For investigating Aag and ADAR1 transcription in splenic cell subsets, spleen cells were additionally stained with anti-CD3 (APC-Cy7, BD Biosciences) and sorted as above. RNA was isolated from sorted cell populations using the RNAqueous-4PCR kit (Ambion).

(ii) PCR amplification and sequencing.
VJ558-rearranged IgH genes were amplified as described previously [23,24] with the published primers, using ~10,000–20,000 cell equivalents of template DNA from germinal center B cells of Peyer's patches, Pfu turbo polymerase (Stratagene), and PCR using 1 cycle at 95 °C for 4 min, 95 °C for 40 s, and 64–58 °C for 40 s (touchdown annealing), 13 cycles at 72 °C for 4 min, followed by 27 cycles at 95 °C for 40 s, 57 °C for 40 s, 72 °C for 4 min, and a final extension at 72 °C for 7 min. J1, J2, J3, and J4 rear-