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Methylation protects cytidines from AID-mediated deamination

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Abstract

Somatic hypermutation (SHM), class switch recombination (CSR), and gene conversion of immunoglobulin genes require activation-induced cytidine deaminase (AID). AID initiates these events by deaminating cytidines within antibody variable and switch regions. The mechanism that restricts mutation to antibody genes is not known. Although genes other than antibody genes have been found to mutate, not all highly transcribed genes mutate. Thus, somatic hypermutation does not target all genes and suggests a mechanism that either recruits AID to genes for mutation, and/or one that protects genes from promiscuous AID activity. Recent evidence suggests that AID deaminates methyl cytidines inefficiently. Methylation of cytidines could thus represent a means to protect the genome from potentially harmful AID activity that occurs outside of the immunoglobulin loci. To test this premise, we examined whether AID could deaminate methylated-CpG motifs in different sequence contexts. In agreement with a report that suggests that AID has processive-like properties in vitro, we found that AID could completely deaminate single-stranded DNA tracks in plasmid substrates that were greater than 300 nucleotides in length. In addition, methylated-CpG motifs, but not their unmethylated counterparts, were protected from AID-mediated deamination. However, methylation did not protect cytidines that neighbored CpG motifs indicating that methylation per se does not provide a more global safeguard against AID-mediated activity. These data also suggest that AID, and possibly other related cytidine deaminases, might represent a more rapid alternative to bisulfite sequencing for identifying methylated-CpG motifs.

Keywords: Activation-induced cytidine deaminase; Somatic hypermutation; Methylation; Bisulfite

1. Introduction

The processes of somatic hypermutation (SHM), immunoglobulin gene conversion (IGC), and class switch recombination (CSR) require the *activation-induced cytidine deaminase* (*AID*) gene (Arakawa et al., 2002; Harris et al., 2002; Martin and Scharff, 2002a; Muramatsu et al., 2000). Mice and humans deficient in AID are incapable of SHM and CSR, while chicken B cells lacking AID cannot carry out IGC. Findings that expression of AID can induce SHM in hybridomas and in non-B cells (Martin et al., 2002; Martin and Scharff, 2002b; Petersen-Mahrt et al., 2002; Yoshikawa et al., 2002) and CSR in fibroblasts (Okazaki et al., 2002) suggest that AID is the only B cell specific protein that is required for each of these processes. It was initially suggested that AID functions by the deamination of a specific mRNA (Muramatsu et al., 2000). However, recent data showing that uridine is an intermediate in the SHM process (Di Noia and Neuberger, 2002; Petersen-Mahrt et al., 2002; Rada et al., 2002) and that AID deaminates cytidines on single-stranded DNA (Bransteitter et al., 2003; Chaudhuri et al., 2004; Chaudhuri et al., 2003; Dickerson et al., 2003; Ramiro et al., 2003; Sohail et al., 2003) supports the notion that AID is a DNA-specific cytidine deaminase.

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It is not known how the process of SHM is targeted to the antibody V-region. Initially, it was thought that only antibody V-regions hypermutated, but investigators found that SHM also targets other genes suggesting that the mechanism for restriction of SHM to antibody genes is not absolute. Mutations caused by the SHM mechanism have been observed in *BCL-6* (Pasqualucci et al., 1998; Pasqualucci et al., 2000; Shen et al., 1998), *Fas* (Muschen et al., 2000), *c-myc* and three other oncogenes (Pasqualucci et al., 2001). However, two highly transcribed genes were not found to mutate (Shen et al., 2000). Thus, targeting of the somatic hypermutation machinery seems to be restricted to a certain degree suggesting a mechanism that either recruits AID to these genes, and/or one that protects the rest of the genome from AID activity.

A recent report suggested that methylated cytidines are poor substrates for AID (Bransteitter et al., 2003). AID exhibited a ∼10-fold reduced specific activity of a methylated-CpG motif than its unmethylated counterpart. Thus, methylation might constitute a mechanism to protect the genome from somatic hypermutation induced by AID outside of the antibody gene loci. Methylation of CpG motifs is generally associated with gene repression. DNA methylation is essential for mouse development and is involved in X inactivation, genomic imprinting, silencing of mobile genetic elements and tumorigenesis (Robertson, 2002). To determine whether methylation of CpG motifs in multiple sequence contexts is refractory to AID-mediated deamination, we analyzed the biochemical properties of AID on long single-stranded DNA substrates that were methylated on CpGs.

2. Materials and methods

2.1. AID purification

AID was expressed as a GST fusion protein in Sf9 insect cells using a baculovirus expression system, as previously described (Bransteitter et al., 2003). GST–AID was batch purified using glutathione-Sepharose (Amersham Pharmacia) and had a purity of ∼75% based on SDS–PAGE. Eluted samples were dialyzed with 20 mM Tris, pH 7.5, 10 mM NaCl, 0.1 mM DTT, 20% glycerol, and stored at −80 °C.

2.2. Bisulfite reaction

Two micrograms of DNA in a volume of 20 μl was denatured by adding 2.2 μl of 3 M NaOH at 37 °C for 20 min. Two hundred and eight microliters of a 6.24 M Urea/4 M sodium bisulfite solution and 12 μl of 10 mM Hydroquinone was then added and run in a thermocycler for 20 cycles of 94 °C for 30 s, 55 °C for 20 min. The DNA was purified using a Qiagen PCR purification kit and eluted in 100 μl of H_2O. The DNA was desulfonated by adding 1/10 volume of 3 M NaOH at 37 °C for 20 min. DNA was ethanol precipitated and dissolved in 10 mM Tris (pH 8.0), 1 mM EDTA.

2.3. Methylation

Ten micrograms of p219 plasmid was methylated using SssI methyl transferase (NEB), which specifically methylates cytosines within CpG motifs. The reaction was carried out in a total volume of 200 μl at 37 °C for 3 h. This reaction was also run in parallel without the methylase enzyme. Both reactions were then purified using phenol–chloroform extraction and ethanol precipitation, and the DNA was dissolved in H_2O. The plasmids were then digested with *Hin*dIII, and the DNA was once again purified. Methylation was verified by digesting both the methylated and unmethylated plasmid DNA with *Msp*I and *Hpa*II.

2.4. AID-deamination reactions

In a typical deamination experiment (20 μl volume), AID (200 ng), RNaseA (1 μg), and DNA substrate (100 nM) were incubated in the reaction buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1 mM DTT) at 37 °C for the indicated time points. Reactions were terminated by incubating at 95 °C for 10 min. The p219 plasmid has the human β-globin promoter. The sequence of the promoter is present in the HUMHBB genebank file, nucleotides 61770-62137. One microliter of the sample was used for the PCR reaction using Taq and the following primers: Primer P1 (EB403-2): GGAAGGTATGAAAATAGGAAAAGAAAATAAATAAATTTTG; Primer P2 (EB319-2): CCCCTAACTTTTATACCCAACCCTAACTCC; Primer P3: (EB318): CCCCCCGGATCCGTATTTTTGGATAGTTAGGTGGT; Primer P4: (EB338): CCCCCCGGATCCAATTAACCAACCCTAAAATATAA. PCR reactions were 30 cycles each using indicated annealing temperatures (Fig. 1). The nested PCR reactions were 30 cycles of 95 °C for 15 s, 58 °C for 15 s,

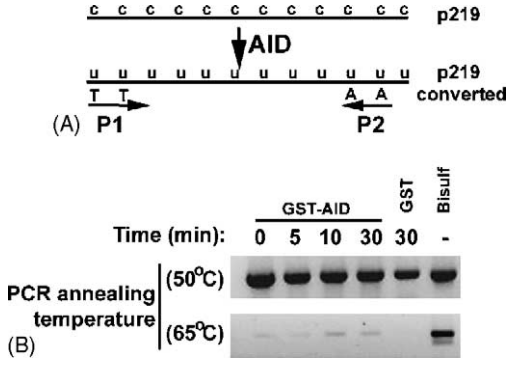

Fig. 1. Amplification strategy to select for products deaminated by AID. (A) Schematic of the β-globin promoter region present within the p219 plasmid and the PCR primers (P1 and P2) to be used for the PCR reaction. P1 and P2 are designed to preferentially anneal with deaminated DNA. (B) PCR amplification of the β-globin promoter region using two annealing temperatures (i.e. 50 and 65 °C) of untreated p219, p219 incubated with purified GST–AID for 5, 10 and 30 min, p219 incubated with purified GST for 30 min, or treated overnight with the bisulfite protocol. PCR products were run on a 1.5% agarose gel, photographed, and shown with the colors inverted.