DNA REPAIR 7 (2008) 77–87

available at www.sciencedirect.com

ScienceDirect

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

Activation-induced deaminase, AID, is catalytically active as a monomer on single-stranded DNA

Sukhdev S. Brar[a,1], *Elizabeth J. Sacho*[b,1], *Ingrid Tessmer*[b], *Deborah L. Croteau*[a], *Dorothy A. Erie*[b,c,*], *Marilyn Diaz*[a,**]

[a] *Laboratory of Molecular Genetics, D3-01, National Institute of Environmental Health Sciences/National Institutes of Health, 111 TW Alexander Drive, Research Triangle Park, NC 27709, USA*

[b] *Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA*

[c] *Curriculum in Applied and Material Sciences, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA*

ARTICLE INFO

Article history:
Received 6 June 2007
Received in revised form
3 August 2007
Accepted 4 August 2007
Published on line 21 September 2007

Keywords:
AID
Deaminase
Monomer
Immunoglobulin
Hypermutation
Switch

ABSTRACT

Hypermutation and class switch recombination of immunoglobulin genes are antigen-activated mechanisms triggered by AID, a cytidine deaminase. AID deaminates cytidine residues in the DNA of the variable and the switch regions of the immunoglobulin locus. The resulting uracil induces error-prone DNA synthesis in the case of hypermutation or DNA breaks that activate non-homologous recombination in the case of class switch recombination. *In vitro* studies have demonstrated that AID deaminates single-stranded but not double-stranded substrates unless AID is in a complex with RPA and the substrate is actively undergoing transcription. However, it is not clear whether AID deaminates its substrates primarily as a monomer or as a higher order oligomer. To examine the oligomerization state of AID alone and in the presence of single-stranded DNA substrates of various structures, including loops embedded in double-stranded DNA, we used atomic force microscopy (AFM) to visualize AID protein alone or in complex with DNA. Surprisingly, AFM results indicate that most AID molecules exist as a monomer and that it binds single-stranded DNA substrates as a monomer at concentrations where efficient deamination of single-stranded DNA substrates occur. The rate of deamination, under conditions of excess and limiting protein, also imply that AID can deaminate single-stranded substrates as a monomer. These results imply that non-phosphorylated AID is catalytically active as a monomer on single-stranded DNA *in vitro*, including single-stranded DNA found in loops similar to those transiently formed in the immunoglobulin switch regions during transcription.

1. Introduction

B cells undergo three mechanisms of somatic gene alteration that profoundly impact the recognition and binding of foreign antigen by antibodies. V(D)J recombination is a developmentally programmed mechanism that generates the diverse pre-immune antibody repertoire. Class switch recombination (CSR) and somatic immunoglobulin (Ig) hypermutation (SHM)

* *Corresponding author at*: Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA. Tel.: +1 919 962 6370; fax: +1 919 962 2388.

** *Corresponding author at*: Laboratory of Molecular Genetics, D3-01, NIEHS/NIH, Research Triangle Park, NC 27709, USA. Tel.: +1 919 541 4740; fax: +1 919 541 7593.

E-mail addresses: derie@unc.edu (D.A. Erie), diaz@niehs.nih.gov (M. Diaz).

[1] These authors contributed equally to this work.

1568-7864/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.dnarep.2007.08.002

are antigen-activated mechanisms that lead to the formation of isotype-switched, high affinity antibodies characteristic of the secondary immune response [1–7]. Both CSR and SHM as well as Ig gene conversion in other species such as rabbit and chicken require the cytidine deaminase, AID [8–12]. AID deaminates cytidines, generating uracils in the DNA encoding the variable and the switch regions of the immunoglobulin locus [13,14]. In CSR, the uracil is removed from the DNA of the involved switch regions by uracil DNA glycosylase (UNG), generating abasic sites [14]. The requirement for at least part of the non-homologous end-joining double-strand break DNA repair machinery in CSR suggests that these abasic sites lead to breaks that trigger the switch reaction [15–17]. Indeed, AID-dependent DNA breaks have been extensively documented for the switch regions [17–22]. In SHM, the resolution of the AID-generated uracil in the DNA is less clear. It appears that hypermutation at G:C base pairs that results from deamination of cytidine residues in the DNA of Ig V regions can originate from replication over the uracil leading to G:C transitions. Alternatively, it can originate from excision of uracil by UNG presumably via a base excision repair mechanism leading to both transitions and transversions at G:C base pairs [14]. Mutations at A:T base pairs, while entirely dependent on AID (AID deficient mice lack mutations from both A:T and G:C base pairs), are likely an indirect result of AID-mediated deamination involving the mismatch DNA repair proteins and translesion synthesis DNA polymerases (TLS) [23–36; for a review see 37]. The requirement for mismatch repair proteins and TLS DNA polymerases for the A:T phase of hypermutation suggests that the G:U mismatch generated by AID initiates a mismatch repair synthesis patch that utilizes the error-prone TLS polymerases instead of the high fidelity DNA polymerases [37]. This model is still speculative since the resolution of the AID-generated uracil in the DNA of Ig V regions leading to mutations at A:T base pairs remains unclear. Finally, it is possible that in addition to the DNA encoding Ig V regions, AID may also deaminate an RNA encoding molecules critical to SHM and CSR [38], although an RNA target has not been identified.

AID deaminates single-stranded but not double-stranded DNA *in vitro* [39–42] except for double-stranded DNA that is actively undergoing transcription [42–45], particularly if AID is phosphorylated by PKA and in a complex with RPA. AID preferentially deaminates microsequences associated with SHM hotspots (WRCY) [39,46–49], providing a rationale for the existence of SHM hotspots that are predominantly found in the complementarity determining regions (CDR's). Data from "pull-down" assays using tagged AID proteins [50] and from structural modeling based on homologous cytidine deaminases [51] suggest that AID may function as a dimer to deaminate DNA. However, it remains unclear whether or not AID is catalytically active as a monomer. Herein, we examine AID deamination of single-stranded DNA alone or embedded within double-stranded DNA in the form of loops. We determined the oligomeric state of the protein by direct visualization using atomic force microscopy (AFM) [52]. Surprisingly, the AFM experiments demonstrate that the dominant AID species is a monomer and that dimerization is not promoted by the presence of single-stranded DNA. These AFM data taken together with a kinetic analysis of deamination suggest that a monomer of AID is sufficient to catalyze the deamination of single-stranded DNA *in vitro*.

2. Methods and materials

2.1. *Preparation of oligonucleotide substrates*

The oligonucleotides used in the deamination assays and the predicted structures of the double-stranded oligonucleotides are depicted in Fig. 1. The "L-oligo" was designed based on the primer used by Yu *et al.* [46] while the single-stranded DNA oligonucleotide (AID1 primer) was based on the CDR2 sequence of the human Ig heavy chain VH3-30-3. Oligonucleotides (100 μM) were 5′-end labeled with γ-^{32}P ATP (3000 Ci/mmol, Amersham Biosciences) using Optikinase (United States Biochemical Corp.) according to the manufacturer's instructions. The reaction was terminated by the addition of 20 mM EDTA, and the enzyme was heat denatured by incubation for 10 min at 65 °C. After labeling, the free nucleotides were removed by gel filtration chromatography (Micro Bio-spin-6, Bio-Rad). For the generation of double-stranded substrates, the labeled oligonucleotide was annealed with the complimentary strand [(AID1 + AID2 = double strand) (AID1 + AID8 = bubble), and (AID1 + AID9 = loop) see Fig. 1] using 1.2 M excess of cold oligonucleotides in the presence of 100 mM KCl, heated at 65 °C for 5 min and slowly cooled to room temperature. The duplexes were analyzed on a native 10% polyacrylamide gel.

2.2. *Deamination assays*

Typically, a reaction mixture (10 μl) containing 2–100 nM radioisotope-labeled oligonucleotide substrate, 5 nM to 2000 nM purified human AID protein (Enzymax, University of Kentucky), 100 ng of RNase A (*Qiagen*) and one unit of uracil DNA glycosylase (Invitrogen) was incubated for 15 min at 37 °C in a buffer containing: 25 mM Tris–Cl, pH 8.0, 50 mM NaCl, and 5 mM EDTA (see scheme in Fig. 2a). C′-terminus $(histidine)_6$-tagged AID protein was purified from *E.coli* by Enzymax (The University of Kentucky, Advanced Science and Technology Commercialization Center) by affinity column (HiTrap Chelating HP™ followed by HiTrap Heparin HP™, GE Healthcare) using an elution buffer consisting of 50 mM Tris (pH of 7.5), 5 mM β-mercaptoethanol, 10% glycerol and 500 mM NaCl. Also, a mutant form of human AID wherein the second cysteine in the active site (position 90) was mutated to alanine was used as a control and it was purified under identical conditions as the wild type AID, by Enzymax. We confirmed the purity of the AID protein preparation using a Coomassie stained gel with SimplyBlue SafeStain (Invitrogen, Carlsbad, USA) per manufacturer's instructions, and in deamination assays, the wild type protein but not the mutant form, deaminated a single-stranded DNA substrate (Supplemental figure). To examine deamination at limiting and excess AID protein concentrations, deamination reactions were carried out at final AID protein concentrations ranging from 5 nM to 2 μM and oligo concentrations ranging from 2 nM to 100 nM. For a subset of samples, RNAse A (400 ng) (Qiagen) was pre-incubated with AID for 30 min prior