First AID (Activation-induced Cytidine Deaminase) Is Needed to Produce High Affinity Isotype-switched Antibodies*

Published, JBC Papers in Press, April 19, 2006, DOI 10.1074/jbc.R600006200

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An answer to the question "why don’t you die when I sneeze?" resides in the ability of higher organisms to mount an aggressive defense in response to an invasion by foreign substances called antigens. In part this defense mechanism is accomplished through the synthesis of high affinity antibody (Ab)

proteins that effectively destroy the attack. Initially, lymphoid B cells rearrange immunoglobulin (Ig) genes using V(D)J recombination to generate a diverse repertoire of low affinity Abs. Subsequently these Ig genes undergo somatic hypermutation (SHM) and class switch recombination (CSR) to generate high affinity Ab molecules of different isotypes. Activation–induced cytidine deaminase (AID) is required to initiate both CSR and SHM. The current understanding of immunological diversification can be attributed to the contributions made by cellular and molecular immunologists with biochemical enzymology recently entering the arena. This minireview discusses advances in the understanding of Ab diversification from a biochemical perspective emphasizing the enzymatic properties of AID in initiating SHM and CSR and exploring the role of downstream mutation fixation events mediated by mismatch repair (MMR), basic excision repair (BER), and error-prone DNA polymerases (EP pols).

Defining Features of SHM and CSR

Activated germinal center B cells mutate and rearrange their Ig genes to produce high affinity Abs of different isotypes using two tightly regulated mechanisms, SHM and CSR. SHM generates point mutations at a rate of $10^{-7}–10^{-8}$ per cell division, about a million times greater than typical somatic cell mutation frequencies (1). The mutations begin at ~200 bp downstream from the Ig gene transcription start site, peak over the variable (V) region, taper off at ~1.5–2 kb downstream from the promoter, and end before reaching the constant (C) region exons (2). Active transcription is required for SHM (3, 4). Mutations are about equally divided between non-transcribed and transcribed strands (2, 5). Of the 50% of mutations that occur at G/C sites, 37% reside within the WRC hot spot motifs (R = A/G; Y = C/T; W = A/T) (6). Of the A:T mutation sites, 34% occur at A sites and 16% at T sites on the non-transcribed strand (5), revealing a strand bias for mutations at A sites on the non-transcribed strand.

SHM is a region-specific recombination event that exchanges the Ig heavy C\textsubscript{H}\textsubscript{m} exon (coding for an IgM antibody) for a downstream C\textsubscript{H} exon, such as C\textsubscript{H}2, C\textsubscript{H}3, or C\textsubscript{H}4, producing IgG, IgA, or IgE antibody isoforms, respectively. Recombined C\textsubscript{H} exons alter the "effector" functions that determine where in the body the Ab resides and how the antigen is destroyed. Double-stranded breaks are generated within the donor switch (S\textsubscript{mu}) region and recipient S region of another downstream target C\textsubscript{H} exon. The C\textsubscript{H} exons between the two break sites form a deleted circular molecule. The VDJ region and a downstream C\textsubscript{H} exon are then joined together by non-homologous end joining (7). S regions range from 2.8 to 12 kb in length, and recombination is region-specific rather than sequence-specific (8).

AID-catalyzed Cytosine Deamination Initiates SHM and CSR

SHM and CSR were thought to be independent events until the discovery of AID (9). AID is both necessary and sufficient to induce SHM and CSR in B cells, hybridoma B cells, and fibroblasts (10, 11), and expression of AID in Escherichia coli induces hypermutation on a target chromosomal gene (12). Previously it was not clear if AID was acting on dsDNA, DNA/RNA hybrids, secondary DNA structures, or RNA. Now, biochemical data show that AID deaminates C on ssDNA (13) and principally on the ssDNA exposed in the non-transcribed strand of dsDNA transcribed by T7 RNA polymerase in vitro (14–16). AID acting on ssDNA substrates explains why transcription is required for both SHM and CSR and how transcription rates affect the rate of SHM mutations. Increased rates of Ig gene transcription provide AID access to more ssDNA on the Ig gene.

Additionally, AID favors C deamination in SHM mutational WRC hot spot motifs while usually avoiding SYC cold spots in accord with in vivo events (14, 16). Some hot spots do not undergo mutation whereas some cold spots are frequently mutated in vivo. Similar phenomenon are seen on artificial substrates incubated with AID in vitro indicating that these mutational events observed in vivo are attributed to the specificity of AID and its random binding to DNA (14, 16).

AID Is Targeted to Ig Loci during Transcription

The "$64,000 question" is how can AID specifically target Ig V and S regions while sparing C regions and non-Ig genes? Transcription alone is insufficient for targeting AID because other highly transcribed genes in B cells are not mutated (17). For the most part, SHM targets are restricted to the Ig loci with the exception of several other loci that acquire SHM-like mutations at a frequency of 10–100-fold less than the Ig loci (11). Ig cis-acting elements, such as the intronic enhancer (iE) and the matrix attachment regions (MARs), have been examined for their effects on SHM. Transcription of V regions is regulated by these enhancer elements, and the rate of transcription correlates with the rate of SHM (4). B-cell-specific transcription factors that bind to these elements could recruit AID to the Ig loci. Elongation factors, such as SII and positive transcription elongation factor b (P-TEFB), are recruited to the pol II elongation complex at ~150 nucleotides downstream from the promoter near the same position where SHM mutations appear (19).

Instead of a factor recruiting AID to the Ig loci, AID may be excluded from non-Ig genes. Recent data from hybridoma experiments report that the cis-acting elements, MARs and iE, may act synergistically to provide AID access to the Ig loci (20). When both elements are present or absent simultaneously on a transgene, SHM rates are unimpaired; however, in the absence of only one element, SHM rates are dramatically reduced (20). The data suggest that these elements acting alone can exclude AID from the Ig loci. Perhaps, one genetic element similar to iE or MARs in a non-Ig loci can block access to AID. An exclusion model could explain how other highly transcribed genes can avoid mutations, whereas genes completely lacking cis-elements, such as a green fluorescent protein reporter gene expressed in fibroblasts, acquire SHM-like mutations (21).

However, there are differences between what occurs on transgenes and on the endogenous Ig loci in vivo. In mice with the iE deleted from endogenous Ig loci, substantial levels of SHM occur, and the MARs alone are not able to exclude AID from the Ig loci (22). Similarly, SHM is impaired when the 3′-E\textsubscript{H} is deleted from transgenes in mice (23), whereas deletion of the 3′-E\textsubscript{H} on the endogenous Ig loci does not dramatically affect SHM (24). These differential effects might be accounted for by the presence of other enhancer elements located proximal to the endogenous Ig genes that can compensate for the loss of the iE.

Reflective of what is observed in vivo, a model T7 transcription system using supercoiled DNA shows a gradient of mutations that occurs downstream from the T7 promoter (16). In contrast to SHM in vivo, the mutation gradient covers a smaller distance (up to 500 base pairs), and the mutations occur mainly on the non-transcribed strand (14–16). Mutations occur on both strands in vivo. Supercoiled regions adjacent to moving...
transcription bubbles could create ssDNA regions on the transcribed strand where AID could act (25). Also, the structure of a chromosomal transcription bubble may allow ssDNA exposure on both strands, whereas this may not occur on a plasmid with T7 transcription. AID deamination occurs on both DNA strands of a chromosomal rif gene in E. coli (12). Although model T7 transcription assays are very useful, they cannot be used to study the effects of human Ig cis-acting elements, such as MARs and the Ig enhancer elements. The future development of an in vitro AID deamination assay coupled with human or mouse RNA polymerase II transcription on Ig genes could be instrumental in revealing direct targeting interactions of AID with Ig elements and transcription factors.

AID Expression and Activity in B Cells Is Tightly Regulated

AID is a B cell-specific enzyme, and its transcription is strictly controlled by PAX5 and E47 (26, 27). Residues predominant in the cytoplasm (Fig. 1). AID is observed in the nucleus in only 3–11% of activated B cells (9, 28). AID contains a nuclear export signal, located in the last C-terminal 10 amino acids (29–31). Deletion of these C-terminal amino acids results in nuclear accumulation of AID (29). Nuclear AID accumulation does not correlate with an increase in mutations in the Ig V or S regions, implying there are additional means to regulate AID access to those regions (29, 32, 33).

Phosphorylation of AID may play an important role in regulating its deamination activity. The catalytic subunit of protein kinase A (PKA) interacts with AID in the cytoplasm (34) and phosphorylates AID at the Ser-38 residue (34, 35), which allows it to interact with replication protein A (RPA) (15). AID phosphorylation at Ser-38 may be required for efficient levels of SHM and CSR. In activated B cells deficient for AID, an S38A mutant restored CSR to approximately 15% of wild-type levels (34, 35). Similarly, other data showed that an S38A AID mutant rescued CSR and SHM activities of AID-deficient cells but with a 60% loss of wild-type AID activity (36). We have determined that AID expressed in baculovirus-infected insect cells is phosphorylated at Ser-38. Phosphorylation at Ser-38 and other sites appears to exert a substantial effect on AID activity because their removal causes about a 80-fold reduction in the rate of deamination on ssDNA.

AID Biochemistry Provides Key Mechanistic Insights Explaining in Vivo Observations

In accord with in vivo data from HIGM-2 patients who have an R24W AID mutation (37), this AID mutant is inactive in vitro on ssDNA (16). A C-terminal deletion of AID that abolishes CSR but does not affect SHM exhibits the same C deamination activity and specificity as wild-type AID acting on ssDNA in vitro (16). AID has been shown to deaminate ssDNA and transcribed dsDNA in a processive manner, perhaps by sliding and jumping (14, 16).

Because of the large positive charge of +11, the N-terminal a-helical region of AID could play a role in regulating the motion of AID along the negatively charged DNA backbone. As anticipated, replacing basic amino acid residues with acidic residues in the N-terminal region reduces the processivity of AID (16), as the average number of deaminations on ssDNA molecules is substantially reduced (16). Multiple AID deaminations on the Ig loci in vivo could ensure that mutations are preserved by overwhelming repair mechanisms. Perhaps more than 10 AID deaminations could be required on the Ig loci before only one escapes proper repair and becomes a permanent mutation. A less processive AID in vivo would likely result in significantly reduced rates of SHM.

The N-terminal AID residues may also influence the structure of the active site. Notably the Arg-35/Arg-36 mutations that reduce processivity and alter deamination specificity are located just two amino acids away from the Ser-38 residue that must be phosphorylated to exert high levels of deamination activity. The mutations at Arg-35/Arg-36 disrupt the PKA phosphorylation consensus sequence (R35R36XSR38). A reduction in processivity could result in fewer mutations and might help to explain why B cells expressing only S38A AID mutants have diminished rates of CSR and SHM.

AID expressed in baculovirus-infected insect cells and in E. coli co-purifies with contaminating RNA molecules that inhibit deamination activity (38). This seemingly adventitious inhibition might, nevertheless, have important biological connotations. For example, as AID is acting on a transcription bubble, it could bind to the RNA being transcribed and become inactivated, which might account for the reduction in deaminations further away from the 3’-end of the promoter. Alternatively, an RNA molecule could bind to AID in the cytoplasm keeping it locked in an inactive form that is unable to enter the nucleus (Fig. 1). A homologous enzyme, APOBEC3G, exists in a high molecular weight RNA complex in activated CD4+ T cells (39). Similar to AID from insect cells, APOBEC3G purified in this complex was inactive unless treated with RNase A (39).

Although a deeper mechanistic understanding of AID awaits high resolution structural analyses of APOBEC family members, recent biochemical studies with AID have led to an important beginning in unraveling the complexities of SHM and CSR. The biochemical data reveal that AID recapitulates several hallmark properties of SHM and CSR: preferential deaminations at C sites with A, T, or G neighbors; strong C-to-T specificity; substantial SHM; and a need for specific amino acid phosphorylation to attain maximal activity.

Error-prone Processing of AID-catalyzed Deaminations during SHM and CSR

AID deaminates C at V and S regions, yet this activity is not sufficient to effect CSR or robust SHM. Rather, it is the processing of AID-induced deaminations that gives rise to mature antibodies (Fig. 2). U-G mismatches are recognized by the MMR complex, MSH2-MSH6, and BER uracil glycosylase...
The U-G mismatch can be processed in a number of ways. Replication over the lesion results in a C→T transition on the newly synthesized strand. Recognition and excision by UDG leads to production of abasic sites that are subsequently excised by BER or MMR proteins, thereby repairing the lesion. Alternatively, UDG may remove U to create a large repair gap. If the gap is filled in by EP pols, C→N and A→N mutations could arise. AID-generated U in V regions may be processed by BER or MMR enzymes to generate dsDNA breaks. The breaks are then repaired by non-homologous end-joining proteins giving rise to an isotype-switched Ig gene.

Biochemical Perspective

Studies on the biochemical properties of AID (how AID distinguishes target C residues in different sequence contexts with good but less than exquisite specificity; its ability to slide and jump processively on ssDNA; the role(s) of phosphorylation; a possible involvement of RNA(s) in regulating AID activity; and the behavior of other proteins working in conjunction with AID, such as RPA, error-prone polymerases, MMR and BER proteins, and cis- and trans-acting transcriptional elements) are essential in determining the molecular mechanisms responsible for Ab diversity. More generally, it is important to establish how AID functions within the recently discovered world of APOBEC nucleic acid deaminases. A. Kornberg’s admonition embodied in his Commandment I (52), “Rely on enzymology to resolve and reconstitute biologic events,” is the timely path to pursue, one that is likely to prove instrumental in revealing the complex secrets of immunology.

Acknowledgment—We express our gratitude to Matthew D. Scharff for critical reading of the manuscript and for providing numerous insightful comments.

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