The Activation-induced Deaminase Functions in a Postcleavage Step of the Somatic Hypermutation Process

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Abstract

Activation of B cells by antigen fuels two distinct molecular modifications of immunoglobulin (Ig) genes. Class-switch recombination (CSR) replaces the Igμ heavy chain constant region with a downstream constant region gene, thereby altering the effector function of the resulting antibodies. Somatic hypermutation (SHM) introduces point mutations into the variable regions of Ig genes, thereby changing the affinity of antibody for antigen. Mechanistic overlap between the two reactions has been suggested by the finding that both require the activation-induced cytidine deaminase (AID). It has been proposed that AID initiates both CSR and SHM by activating a common nuclease. Here we provide evidence that cells lacking AID, or expressing a dominant negative form of the protein, are still able to incur DNA lesions in SHM target sequences. The results indicate that an intact cytidine deaminase motif is required for AID function, and that AID acts downstream of the initial DNA lesions in SHM.

Key words: somatic hypermutation • AID • DNA double-strand breaks • B lymphocyte • Ig gene

Introduction

One of the hallmarks of the immune system is the ability to recognize large numbers of antigens. In the mouse and human B cell repertoires, diversity is generated by two distinct processes; the primary repertoire is generated in the bone marrow by V(D)J recombination (1). Its further diversification involves the antigen-driven introduction of point mutations into the V regions of Ig genes (1). This process of somatic hypermutation (SHM) gives rise to families of related mutant antibodies which are then selected for their binding affinity to the immunizing antigen (2).

During hypermutation, the V regions of Ig genes accumulate single nucleotide substitutions and occasional insertions and deletions. Many of the mutations occur at specific residues (hotspots), suggesting that the sequence surrounding a hotspot might be the target of a putative mutator complex (3). Yet whenever the V gene has been replaced with artificial substrates, those substrates hypermutate successfully (3), and so the sequence of the V gene itself does not initiate the mutation process. Surprisingly, the specific V region promoter can be replaced by heterologous promoters without a detrimental effect on hypermutation (3), but the process is absolutely dependent on the presence of the Ig enhancers. Because of this reliance on promoter and enhancer elements, it is has been suggested that the targeting step of hypermutation is linked to transcription, although whether it is coupled to the transcription process per se or to a general requirement for locus accessibility remains unclear (4–6).

SHM was originally hypothesized to be a two-step process, initiated by cleavage of the DNA within the mutating region, and subsequently resolved by error-prone repair (7). There is strong circumstantial evidence to suggest that the cleaved DNA intermediate is a DNA double-strand break, as V regions of hypermutating cells incur DSBs on hotspots and at high rates (8, 9). Furthermore, the initiation of DSB formation has been shown to require the same elements which regulate the introduction of mutations (transcription and the presence of the enhancer) (8, 9). These hypermutation-associated DSBs are abundant in the G2 (postreplicative) phase of the cell cycle, and thus it has been suggested that they are repaired by homologous recombination between sister chromatids (8).

Even though it has been extensively studied, little is known about the molecular mechanism of the SHM process. The only known protein whose loss of function leads to a significant downregulation (if not total ablation) of hypermutation is the recently discovered activation-induced cytidine deaminase (AID) (10, 11). Yet, the function of

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AID in somatic hypermutation is far from clear, and its effect might not even be direct, as AID is thought to be an RNA-editing enzyme (12). It has been postulated that AID edits the mRNA of the endonuclease responsible for the DNA lesions in both CSR and SHM (10). Alternatively, AID might be responsible in editing the mRNA of a factor (or factors) responsible for orchestrating DSB repair. Here we report that SHM-associated DSBs are still present in cells lacking AID, or expressing a dominant negative form of the protein. Our results indicate that AID acts downstream of the initial DNA lesions in SHM, possibly by editing the RNA of a repair factor.

Materials and Methods

Plasmid Constructs. The AID cDNA was amplified from Ramos cDNA and the H56R/E58Q mutations in AID were introduced with the QuikChange™ kit (Stratagene). To generate the bacterial expression vectors, AID or AID DN were independently cloned into pET3d in frame with GST, to generate the wild-type GST-AID or GST-AID DN fusion gene, respectively. The proteins were expressed in BL21 λDE3 cells and purified with glutathione sepharose 4B resin, according to the manufacturer's instruction (Amersham Pharmacia Biotech).

To generate the retroviral expression vectors, the AID DN cDNA was cloned into the pMSCV retroviral expression vector (CLONTECH), engineered to express either GFP or a puromycin resistance gene from an internal phosphoglycerol kinase (pgk) promoter.

Mice and Cell Culture. AID$^{-/-}$ (10) mice have been described previously. The Ramos (RA-1) cell line was obtained from the American Type Culture Collection and grown as described previously (13). For retroviral infections, pMSCV or pMSCV-AID DN, along with the packaging plasmid pkat (14), were cotransfected into 293T cells by calcium phosphate precipitation and viral supernatants harvested 48 h after transfection were used to infect cells.

Deamination Assay. The deamination assay was performed as described previously (15). Briefly, 100 μg of protein was added to a mix containing 25 μmol Tris-HCl, pH 7.05, and 1 μmole cytidine (1 ml final volume). At different times, 100 μl samples were added to 900 μl of 0.5 M ice-cold perchloric acid and the mixture was centrifuged. The absorbance of the supernatant was measured at 290 nm. Under these conditions, the difference in the molar extinction coefficient between cytidine and uridine is 10.1×10^6 cm^2 per mol (15).

LM-PCR and RT-PCR. Cells were embedded in agarose and genomic DNA prepared as described previously (8). The sequences of gene specific primers not described in (8) were as follows: mouse VH186.2, VH186.2 L1 TTCTTGGCAGCAA-CAGCTACAGGTAAGG VH186.2 L2 GCAGGCTTGAG-GTCTGGACATATACATG probe GACATCCACTTTGC-CTTTCTCTCCACAGGTG; mouse Vλ1, Vλ1 L1 GTT-GTGACTCAGGAATCTGCACTCACC Vλ1 L2 GGTGAAA-CAGTCACACTCACTTGTCGC probe GTACTGGGGGCT-GCTACAACTAGTAACTATGCCAACTG; and mouse Cμ, Cμ L1 GCTTCCCATCAGTCCTGAGAG Cμ L2 CCACCT-CACAGGTGCTGCTGCCTTCC probe CACGTGGTGTG-CAAAGTCCAGCACC. Total Ramos RNA was prepared using RNAsol (Tel-Test, Inc.), treated with DNase, and reverse transcribed using random nonamers. c-myc, and human AID were PCR amplified (30 cycles) from cDNA from 10^5 cells using hot-start Taq (QIAGEN) and combinations of the following primers: human AID, hAID left GTCCGCTGGGCTAAGGGTCGGCG hAID right GCACCCCGGCGCGGTGCAGCCG; and human c-myc, c-myc left CCGCCCGCGGCCACAGCGTC c-myc right CGCCTCTTGACATTCTCCTCGGTGTC.

Flow Cytometry and Cell Sorting. To purify germinal center B cells, we pooled spleens from mice at 14 d after immunization with NP-CGG in alum (Biosearch Technologies, Inc.), and separated the resting and activated lymphocytes on a Percoll gradient (Amersham Pharmacia Biotech). Cells were stained with the pan-B cell marker B220 (phycoerythrin) and the B/T activated cell marker GL-7 (FITC) (both from BD PharMingen), and sorted on a Becton Dickinson FACSVantage™. Sorted cells were immediately embedded in agarose for further analysis. For the cell cycle study, standard methods were used (16). All analyses of flow cytometric data were performed using the FlowJo™ software package (Treestar).

Results

An AID Catalytic Mutant Has No Deaminase Activity. The primary sequence of AID shares highest sequence similarity with Apobec-1 (12) (Fig. 1 A), the prototypic RNA-editing cytidine deaminase (14). Cytidine deaminases in general share a conserved active site motif consisting of a histidine and two cysteines which coordinate Zn^{2+}, and a glutamate which serves as a proton donor in the deamination reaction (17). Replacement of any of these four amino acids results in a catalytic mutant (18). We generated an AID protein mutated in two of these residues (H56R/E58Q) and expressed both the mutant and wild-type proteins in *Escherichia coli*. The wild-type protein was active in an in vitro deamination assay while the catalytic mutant was not (Fig. 1 B).

All except one of the known cytidine deaminases act as homodimers or homotetramers (19), and hence catalytic mutants can have a dominant negative effect (19, 20). A series of leucines in the carboxy terminus of Apobec-1 is thought to be important for dimerization (20), and even though there is low sequence homology between AID and Apobec-1 in that region, these leucines are conserved in AID. Furthermore, secondary structure prediction algorithms (21) model the carboxy terminus as almost entirely α-helical with a potential for coiled coil formation (Fig. 1 C). Hence the H56R/E58Q mutant AID (henceforth AID DN) might display a dominant negative phenotype.

AID DN Interferes with SHM. To determine the effect of the putative dominant negative protein on SHM, we expressed the protein in the human Burkitt lymphoma cell line Ramos, which hypermutates constitutively in culture (13). Cells were infected with a retrovirus expressing human AID DN from the long terminal repeat promoter and a puromycin resistance gene (puro) from an internal pgk promoter. Infected cells selected with puromycin expressed AID DN at levels 20-fold higher than endogenous AID, as assessed by semiquantitative RT-PCR (Fig. 2 A). As controls, cells were infected with retroviruses expressing only the puro gene (designated "empty") or wild-type AID in addition to puro. Infected cells (puromycin resistant) were

A

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AID:     56  HVELLFLR-YISDWDLDPGRCYRVTWFTSWSPCYDCARHVADFLRGNPNLSLRIFTARLY 114
             |||+ |++ + |+ |     |      +|||  ||||||++| ++ + ||   +| ++|  |+ |||+
Apobec:  61  HVEVNFIKKFTSERDFHPSISCSITWFLSWSPCWECSQAIREFLSRHPGVTLVIYVARLF 120
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B

C

Figure 1. Characterization of recombinant AID proteins. (A) Alignment of the predicted active site of AID and the active site of Apobec-1. Conserved catalytic residues are indicated in red. (B) Cytidine deamination assay. Recombinant GST-AID wild-type (WT) and GST-AID DN mutant were expressed in and purified from bacteria. CTP was incubated with purified GST-AID WT, GST-AID DN, or BSA, and the amount of substrate remaining at different time points was determined by absorption at 290 nm. The experiment was repeated with AID DN tagged with a minimal tag (strep tag, Cell Genesys) with similar results (data not shown). (C) Secondary structure prediction for human AID. C (gray) indicates potential loops (or nonstructured regions), E (yellow) indicates a β-sheet, and H (blue) indicates an α-helix (modeling was done on the 3D-PSSM server; reference 21).

seeded at single cell density, and after 6 wk of expansion, the Ig heavy chain variable region was PCR amplified, cloned, and sequenced. We assayed six clones infected with the empty retrovirus, and in all cases, 4–6 independent mutations were identified (Fig. 2 B), yielding a mutation frequency of 0.5×10^{-5}/bp/generation (calculated as per Capizzi and Jameson; for a review, see reference 22). The results from the analysis of clones infected with wild-type AID retrovirus were comparable (data not shown). In contrast, Ramos clones expressing AID DN contained virtually no mutations (Fig. 2 B), yielding a mutation frequency at least 20-fold lower than that of cells infected with the empty virus (0.02×10^{-5}/bp/generation). We conclude that expression of AID DN suppresses SHM (presumably by interfering with the function of the wild-type protein), yielding a phenotype functionally equivalent to full AID deficiency. The results also indicate that an intact cytidine deaminase catalytic motif (and therefore deaminase activity) is essential for the function of AID.

SHM-associated DSBs Are Not Affected by Expression of AID DN. Ramos cells were infected with retroviruses that expressed either puromycin alone (empty virus), or puromycin in conjunction with wild-type AID or AID

DN, selected in the antibiotic, and assayed for DSBs in the Ig heavy and light chain variable regions. Consistent with our previous report (8), we can titrate the number of linker-ligated genomes down to 50 and still detect DSBs in Ramos cells infected with the empty virus or with the wild-type AID virus (Fig. 3 A and data not shown). Ramos cells infected with the AID DN retrovirus continue to in-

Figure 2. AID DN interferes with SHM. (A) RT-PCR analysis of AID expression levels in wild-type Ramos cells (left) and Ramos cells transduced with an AID-DN–expressing retrovirus (right). Amplification of c-myc controls for input cDNA. (B) IgH variable region mutations per culture after 6 wk of expansion (based on 48 sequences per culture). Cultures were grown from single Ramos cells transduced with empty virus or with AID-DN–expressing virus.

cur DSBs over the Ig heavy variable region, at a similar or even slightly higher frequency than uninfected cells or those infected with the empty virus (bands are reproducibly detected with as few as 10 input genomes; Fig. 3 A and data not shown). Similar results were obtained for the Igλ vari-
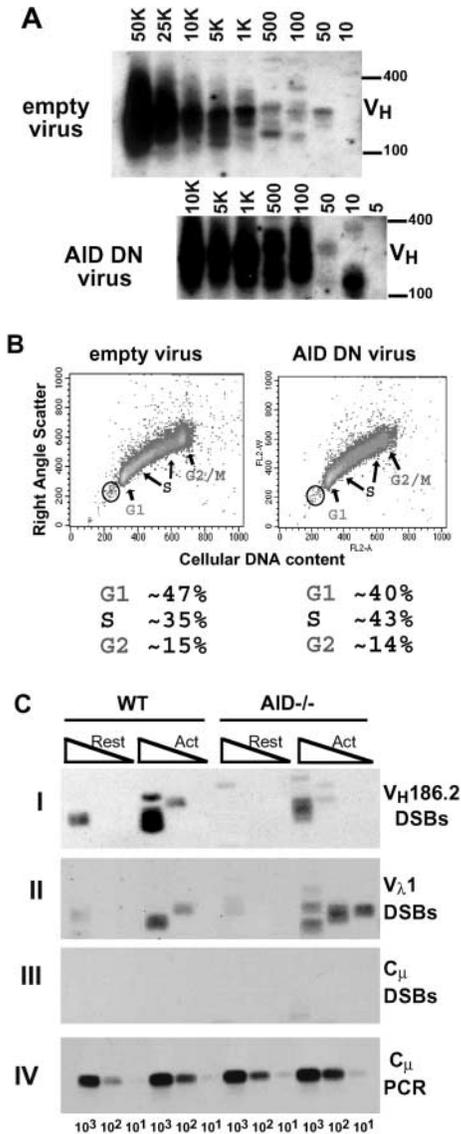

Figure 3. SHM-associated DSBs are not abrogated by AID DN or AID deficiency. (A) LM-PCR amplification of DSBs from the endogenous V_H region of Ramos cells infected with empty virus or with AID DN virus. Genome equivalents of linker-ligated template DNA are indicated above the lanes, and the size of molecular weight markers to the right (bp). (B) Cell cycle status analysis of retrovirally infected Ramos cells using propidium iodide staining. The percentage of cells in G1, S, and G2 are indicated below the FACS® plots. The circle indicates the expected location of apoptotic cells. (C) LM-PCR amplification of DSBs from the $V_H186.2$, Vλ1, and Cμ genes from C57BL/6 (WT) or AID$^{-/-}$ mice 14 d after immunization with NP-CGG. Resting (B220$^+$, GL7$^-$, small) and activated (B220$^+$,GL7$^+$, large) splenic B cells were compared. Genome equivalents of linker-ligated template DNA are indicated below the lanes. Standard PCR of Cμ (bottom panel) indicates that equivalent amounts of DNA were used for each PCR reaction.

able region (data not shown). Thus, interference with AID function through expression of AID DN does not diminish the amount of DSBs over the Ramos variable regions, even though it dramatically reduces mutation accumulation.

DNA DSBs can be lethal to the cell if not repaired. Since the introduction of mutations is thought to occur during the repair of SHM-associated DSBs (8, 9), it was possible that Ramos cells expressing AID DN (which have abundant DSBs but very few mutations) would exhibit substantial cell death. To investigate this possibility, we stained Ramos cells with propidium iodide and analyzed them for DNA content. The percentage of cells in the G1, S, and G2 phases of the cell cycle did not vary significantly between Ramos cells infected with empty virus, wild-type AID or the AID DN virus (Fig. 3 B and data not shown). Importantly, no subdiploid peak indicative of dead cells was observed in any of the cultures. Furthermore, neither the proliferation rate nor the restriction of DSBs to the late S/G2 phase of the cell cycle (8) was affected by AID DN expression (data not shown). Thus, Ramos cells infected with the AID DN retrovirus do not die as a result of unresolved DSBs, indicating that these DSBs are repaired but predominantly in an error-free fashion.

SHM-associated DSBs Are Not Affected by Deletion of AID. Our data suggest that ablation of the catalytic activity of AID results in a dominant negative phenotype with respect to SHM, but does not impair the formation of SHM-associated DSBs. It is possible, however, that AID has functions that do not depend on the cytidine deaminase activity (for instance in facilitating DSB formation). To examine this possibility, we assessed Ig variable region DSBs in activated B cells lacking AID. Wild-type and AID$^{-/-}$ C57BL/6 mice were immunized with NP-CGG, an antigen preferentially recognized by a specific heavy and light chain combination ($V_H186.2$ and Vλ1) (23). At 14 d after immunization, splenic B cells were collected and sorted into germinal center (GC) and resting B cell populations. We could reproducibly amplify DSBs over the $V_H186.2$ and Vλ1 genes both from wild-type and AID$^{-/-}$ cells (Fig. 3 C and data not shown). Such DSBs could be amplified from both the GC and the resting B cell fractions, but were preferentially present in the GC B cell population (10- to 100-fold higher frequency, Fig. 3 C, panels i and ii). The observed DSBs were V region specific since no DSBs could be amplified from the Ig μ constant region or from the recombination activating gene (RAG)-1 gene (Fig. 3 C panel iii, and data not shown). We conclude that in the absence of AID, SHM-associated DSB formation remains intact both in vitro and in vivo, and therefore, that AID likely functions in a postcleavage step of the SHM process.

Discussion

The immune system generates diversity by using three distinct mechanisms of genetic alteration: V(D)J recombination, CSR, and SHM. Mechanistically, all three processes can be divided into at least three phases: targeting/

recognition, DNA cleavage, and repair (18). In all three cases, transcription is thought to play a key role in targeting of a nuclease to the Ig locus. The cleavage step is thought to result in the production of a DNA DSB, though the creation of the DSB might not be the initial event. In V(D)J recombination for instance, the initial event is the nicking of one strand by the RAG recombinase, followed by hairpin formation and resolution into a blunt DSB (24). It is not known whether the CSR nuclease initially inflicts a single or double-strand break but there is some evidence to suggest that the final product of cleavage is a DSB (25). Finally, there is data to indicate that SHM also involves the creation of a DNA DSB, though that may not necessarily be the first step of the cleavage reaction. Indeed, it is possible to amplify single-strand ends from hypermutating sequences (26), though whether the experiments in (26) detect one of the two ends generated by a DSB or alternatively, the unique end of a single-strand break remains unclear. Thus, regardless of the particular mechanics which lead to the creation of the DSB, the end product of the cleavage step of the reaction and hence, the substrate upon which DNA repair must act, in all three cases is likely to be a DNA DSB.

In vertebrates, the two main pathways of DSB repair are homologous recombination and nonhomologous end joining (NHEJ). These appear to operate predominantly in distinct phases of the cell cycle: G_1/early S for NHEJ and late S/G_2 for homologous recombination (27). The DSBs associated with V(D)J recombination are repaired by NHEJ and are found almost exclusively in G_1 phase cells. There is evidence to suggest that NHEJ might be the repair mechanism of choice for CSR-generated ends (25). In contrast, SHM-associated DSBs are found in G_2 phase cells and it has been postulated that they are repaired by homologous recombination (8, 28). Yet, while SHM and CSR have been proposed to involve distinct repair pathways, the end products of CSR (the switch joints) incur point mutations at high frequency (29) suggesting either that a common error-prone polymerase is involved in class–switch–associated NHEJ repair as well as in SHM-associated recombinational repair or that CSR repair requires both the NHEJ and the homologous recombination machinery.

Mechanistic overlap between CSR and SHM is further suggested by the finding that both reactions require AID (10). As ablation of AID leaves the targeting stage (the generation of germline transcripts) of CSR intact (10), AID has been proposed to function either at a pre or postcleavage step. In an attempt to distinguish whether AID is responsible for editing the nuclease or for coordinating repair, Nussenzweig and colleagues have looked at the association of repair factors with switch region DNA in wild-type or AID$^{-/-}$ mice (30). The repair factors Nbs1 and histone γH2AX are known to accumulate in high concentrations over areas which harbor DNA breaks, where they form distinct nuclear "foci." Such foci form readily over switch regions in LPS-IL-4–stimulated wild-type B cells which incur DNA breaks in the process of CSR, but are absent in similarly stimulated, AID-deficient B cells (presumably because of the lack of DSB formation in the absence of AID). Therefore in CSR, AID may act upstream of repair and perhaps functions in initiating DSB formation (30).

If SHM and CSR were two different repair outcomes of the similar DNA cleavage reactions as suggested previously (30), the expectation would be that DNA lesions would be absent in hypermutating sequences in AID-deficient cells. Our results, however, provide evidence that cells lacking AID, or expressing a dominant negative form of the protein, are still able to incur DNA lesions in SHM target sequences, which can be amplified and detected by ligation-mediated PCR. Ramos cells overexpressing AID DN, and germinal center B cells from AID$^{-/-}$ mice, incur DSBs in their Ig V regions without accumulating mutations (Fig. 3). Furthermore, Ramos cells expressing AID DN do not die, nor is their cell cycle perturbed, leading to the conclusion that they must repair the abundant DSBs efficiently and accurately (Fig. 2). Since AID$^{-/-}$ B cells are still able to incur DNA DSBs over hypermutating sequences, either AID plays different roles in SHM and CSR (for instance, by editing two separate mRNAs), or that DSBs are not mechanistically linked to the SHM reaction. There is little doubt that DSBs over hypermutating sequences are associated with the process; they only occur over sequences which hypermutate, they are extremely abundant, they coincide with mutational hotspots, and they are present in the phase of the cell cycle in which repair of SHM lesions probably occurs (10, 11, 13). However, it is possible that SHM is initiated by the introduction of single-strand nicks which get converted into mutations, and that the DSBs are formally not intermediates but rather byproducts of that reaction (18). Even then, the fact that DSBs are abundant over hypermutating sequences in AID-deficient cells argues strongly that the formation of the initial lesion (be it a nick or a DSB) as well as its potential byproducts is not dependent on the function of AID. Thus, though both CSR and SHM employ AID, they might do so in different steps of the two reactions.

We conclude that AID deficiency impairs SHM, but does not affect the creation of the DNA breaks associated with this process. Therefore, it is most likely that AID plays a role in the postcleavage (repair) phase of this reaction. Its action may transiently alter a DNA repair factor, thereby creating an error-prone repair machinery capable of resolving SHM DNA lesions. Our experiments further suggest that the cytidine deaminase catalytic core of AID is crucial for its role in SHM. Thus if AID is an RNA-editing deaminase, its role in SHM is most likely to edit the RNA of a DNA repair factor.

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