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 Igα 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
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 DSBR repair. Here we report that SHM-associated DSBRs 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 DE3 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 pM-SCV-AID DN, along with the packaging plasmid pkat (14), were described previously (15). Briefly, 100 μl of 0.5 M ice-cold perchloric acid and the mixtures were centrifuged. The absorbance of the supernatant was measured at 290 nm. Under these conditions, the difference in molar extinction coefficient between cytidine and uridine is 10.1 × 10^6 cm^2 mol^{-1} (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 TTCTTGGCAGCACA-CAGCTACAGGTAAGG VH186.2 L2 GCAGGCTTGGAG-CTCTGGACATATACAGT probe GACATCCACTTTGCCTTCTCCTACAGGTTG; mouse Vα1, Vα1 L1 GTTCAGTGGGAGATCTCTACACCAV1 L1 GTGGAAGACYCCTACACTCTGTGGC probe GTACTGGGCGTGCTGACTAAGCTATATGGTTACACCT; and mouse Cp, Cp L1 GTCTCCCATCGCTCTGGGAG Cp L2 CCACCTCAGGCTTGGCTGCCCTCC probe ACAGGCTTGGGTGAAGCTTAACTACAGGTC.
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 \(0.5 \times 10^{-3}/bp/generation\) (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 \((0.02 \times 10^{-3}/bp/generation)\). 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-
The Role of AID in Hypermutation

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 Igα vari-

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 unre-

olved 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 exam-

ine 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 (VH186.2 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 VH186.2 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 VH region specific since no DSBs could be amplified from the Igλ 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 recombina-

tion, 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: G1/early S for NHEJ and late S/G2 for homologous recombination (27). The DSBs associated with V(D)J recombination are repaired by NHEJ and are found almost exclusively in G1 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 G2 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 mechanismically 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.

We are grateful to T. Honjo for generously providing the AID−/− mice. We thank T. Taylor and G. Tokmoulina for help with cell sorting and E. Hilton for special assistance with DNA sequencing. Oligonucleotide synthesis and DNA sequencing were performed by the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

F.N. Papavasiliou is an Irene Diamond Assistant Professor and D.G. Schatz is an Associate Investigator of the Howard Hughes Medical Institute.
References

1. Rajewsky, K. 1996. Clonal selection and learning in the antibody system. Nature. 381:751–758.
2. Weigert, M.G., I.M. Cesari, S.J. Yonkovich, and M. Cohn. 1970. Variability in the AluII chain sequences of mouse antibody. Nature. 228:1045–1047.
3. Neuberger, M.S., M.R. Ehrenstein, N. Klix, C.J. Jolly, J. Yelamos, C. Rada, and C. Milstein. 1998. Monitoring and interpreting the intrinsic features of somatic hypermutation. Immunol. Rev. 162:107–116.
4. Peters, A., and U. Storb. 1996. Somatic hypermutation of immunoglobulin genes is linked to transcription initiation. Immunity. 4:57–65.
5. Goyenechea, B., N. Klix, J. Yelamos, G.T. Williams, A. Riddell, M.S. Neuberger, and C. Milstein. 1997. Cells strongly expressing Ig(κ) transgenes show clonal recruitment of hypermutation: a role for both MAR and the enhancers. EMBO J. 16:3987–3994.
6. Fukita, Y., H. Jacobs, and K. Rajewsky. 1998. Somatic hypermutation in the heavy chain locus correlates with transcription. Immunity. 9:105–114.
7. Brenner, S., and C. Milstein. 1966. Origin of antibody variation. Nature. 211:242–243.
8. Papavasiliou, F.N., and D.G. Schatz. 2000. Cell-cycle-regulated DNA double-stranded breaks in somatic hypermutation of immunoglobulin genes. Nature. 408:216–221.
9. Bross, L., Y. Fukita, F. McBlane, C. Demolliere, K. Rajewsky, and H. Jacobs. 2000. DNA double-strand breaks in immunoglobulin genes undergoing somatic hypermutation. Immunity. 13:589–597.
10. Muramatsu, M., K. Kinoshita, S. Fagarasan, S. Yamada, Y. Shinkai, and T. Honjo. 2000. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell. 102:553–563.
11. Levy, P., T. Muto, Y. Levy, F. Geissmann, A. Plebani, O. Sanal, N. Catalan, M. Forveille, R. Dufourcq-Labelouse, A. Gennery, et al. 2000. Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the hyper-IgM syndrome (HIGM2). Cell. 102:565–575.
12. Muramatsu, M., V.S. Sankaranand, S. Anant, M. Sugai, K. Kinoshita, N.O. Davidson, and T. Honjo. 1999. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. J. Biol. Chem. 274:18470–18476.
13. Sale, J.E., and M.S. Neuberger. 1998. TdT-accessible breaks are scattered over the immunoglobulin V domain in a constitutively hypermutating B cell line. Immunity. 9:859–869.
14. Chester, A., J. Scott, S. Anant, and N. Navaratnam. 2000. RNA editing: cytidine to uridine conversion in apolipoprotein B mRNA. Biochim. Biophys. Acta. 1494:1–13.
15. Neuhard, J. 1968. Pyrimidine nucleotide metabolism and pathways of thymidine triphosphate biosynthesis in Salmonella typhimurium. J. Bacteriol. 96:1519–1527.
16. Coligan, J.E., A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober. 2000. Fluorescence activated cytometry. In Current Protocols in Immunology. J. Wiley and Sons, New York.
17. Scott, J., N. Navaratnam, and C. Carter. 1999. Molecular modelling of the biosynthesis of the RNA-editing enzyme APOBEC-1, responsible for generating the alternative forms of apolipoprotein B. Exp. Physiol. 84:791–800.
18. Navaratnam, N., S. Bhattacharya, T. Fujino, D. Patel, A.L. Jarmuz, and J. Scott. 1995. Evolutionary origins of apoB mRNA editing: catalysis by a cytidine deaminase that has acquired a novel RNA-binding motif at its active site. Cell. 81:187–195.
19. Carlow, D.C., C.W. Carter, Jr., N. Mejilhede, J. Neuhard, and R. Wolfenden. 1999. Cytidine deaminases from B. subtillis and E. coli: compensating effects of changing zinc coordination and quaternary structure. Biochemistry. 38:12258–12265.
20. Oka, K., K. Kobayashi, M. Sullivan, J. Martinez, B.B. Teng, K. Ishimura-Oka, and L. Chan. 1997. Tissue-specific inhibition of apolipoprotein B mRNA editing in the liver by adenovirus-mediated transfer of a dominant negative mutant APOBEC-1 leads to increased low density lipoprotein in mice. J. Biol. Chem. 272:1456–1460.
21. Kelley, L.A., R.M. MacCallum, and M.J. Sternberg. 2000. Enhanced genome annotation using structural profiles in the program 3D-PSSM. J. Mol. Biol. 299:499–520.
22. Capizzi, R.L., and J.W. Jameson. 1973. A table for the estimation of the spontaneous mutation rate of cells in culture. Mutat. Res. 17:147–148.
23. Bothwell, A.L., M. Paskind, M. Reth, T. Imanishi-Kari, K. Rajewsky, and D. Baltimore. 1981. Heavy chain variable region contribution to the NPB family of antibodies: somatic mutation evident in a γ2a variable region. Cell. 24:625–637.
24. Fugmann, S.D. 2001. RAG1 and RAG2 in V(D)J recombination and transposition. Immunol. Rev. 23:23–39.
25. Stavnezer, J. 2000. Molecular processes that regulate class switching. Curr. Top. Microbiol. Immunol. 245:127–168.
26. Kong, Q., and N. Maizels. 2001. DNA breaks in hypermutating immunoglobulin genes: evidence for a break-and-repair pathway of somatic hypermutation. Genetcs. 158:369–378.
27. Takata, M., M.S. Sasaki, E. Sonoda, C. Morrison, M. Hashimoto, H. Utsumi, Y. Yamaguchi-Iwai, A. Shinoara, and S. Takeda. 1998. Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. EMBO J. 17:5497–5508.