Commentary

The Function of AID in Somatic Mutation and Class Switch Recombination: Upstream or Downstream of DNA Breaks

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The immune system has evolved specific mechanisms to combat a potentially limitless number of foreign pathogens using a limited arsenal of Ig genes. To diversify the coding potential of the Ig genes, B cells undergo several processes of regulated genetic alterations. Early in their development, B cells in the bone marrow undergo V(D)J recombination to juxtapose variable region V, D, and J segments in different combinations, creating a large repertoire of antibodies (1). Later in B cell development, usually after antigen-dependent activation of B cells, the genetic alteration processes of somatic mutation (SM), class switch recombination (CSR), and gene conversion further diversify the antigen-recognition repertoire as well as the effector function of encoded antibodies. In SM, which is the dominant means of secondary alteration of variable region gene sequences in humans and mice, mutations are introduced in the Ig variable region genes at a tremendous rate, which allows for evolution of high affinity antibodies (2). In some vertebrates, such as chickens and pigs, diversification of assembled Ig variable regions occurs by a gene conversion mechanism rather than SM (3). In CSR, to diversify the effector function of specific antibodies, recombination occurs within the downstream portion of the IgH locus to join variable region genes with different constant (C_H) region genes (4).

SM introduces mutations, small deletions, and insertions at a high rate in a ~2 kb region downstream of the Ig promoter, altering the specificities of the encoded antibodies (2). SM usually occurs within the specific microenvironment of germinal centers, which is thought to be critical for this process. Within germinal centers, antibodies with high affinity for antigen are then selected, while low-affinity antibodies are weeded out in a process termed affinity maturation. The SM mutations commonly occur at conserved sequence motifs (hotspots). The mechanism of SM has been proposed to involve generation of DNA breaks followed by a repair process that involves an error-prone polymerase (5). In gene conversion, the assembled variable region sequences are altered via homologous recombination using other unrearranged variable region genes or pseudogenes as templates.

DNA breaks that occur during SM were first detected by overexpressing the enzyme terminal deoxynucleotidyl transferase (TdT), which catalyzes nontemplated addition of nucleotides to free DNA ends, in a constitutively hypermutating B cell line (6). This study revealed that nucleotides were specifically inserted at SM hotspots, suggesting that these hotspots were sites of DNA breaks. Subsequently, three groups investigated the nature of these breaks (single versus double-strand breaks, blunt versus staggered or hairpin ends) using ligation-mediated PCR (LM-PCR) strategies. These studies detected blunt-ended double-stranded DNA breaks (DSBs) preferentially at hotspot sequences in B cells undergoing SM (7, 8). Papavasiliou and Schatz further showed that the majority of the DSBs were detected during the G2 cell cycle phase when the homologous recombination repair pathway is dominant (7), while Bross et al. demonstrated the reliance of these breaks on transcriptional activity (8). In addition, Kong and Maizels also detected single-stranded DNA breaks at hypermutation sites (9). Although breaks in genomic DNA can arise by a number of mechanisms, including apoptotic DNA fragmentation and in vitro shearing, these studies provided considerable evidence that the breaks detected by the LM-PCR assays were associated with the SM mechanism. Specifically, they showed that the breaks occurred preferentially at SM hotspots, were dependent on transcription, and occurred preferentially in hypermutating B cells.

CSR is another genetic modification employed by B cells to boost the immune response by changing the constant region of the antibody while retaining the antigen-specific variable region. This DNA recombination event occurs between two switch (S) regions, consisting of stretches of repetitive sequence, located just upstream of each C_H gene (except Cδ). CSR is a recombination/deletion mechanism that juxtaposes a downstream C_H (Cγ, Cε, or Cα) to the expressed V(D)J segment, allowing switching from expression of IgM to IgG, IgA, or IgE (4). In vivo, CSR requires germline transcription of S region sequences, the generation of DSBs within S regions, and resolution of these breaks by a process that requires NHEJ factors (4). It has been suggested that CSR may, like SM, involve DNA

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synthesis by an error-prone polymerase, because mutations have been detected near recombination junctions (10).

Recently, the discovery that the AID gene is required for SM, gene conversion, and CSR has linked the mechanisms of these three processes (11–14). AID encodes a cytidine deaminase and shares sequence homology to the RNA editing gene APOBEC-1 (15). Although cytidine deaminase activity has been demonstrated for AID in vitro, neither the function nor the substrate of AID is known. Like APOBEC-1, AID could edit an RNA transcript to change the function of the encoded protein, for example, an endonuclease or error-prone polymerase. Another possibility is that AID acts on relevant RNA or DNA sequences, targeting them for recombination or mutation. To date, there is no evidence for either possibility. The function of AID has been studied in light of the three general requirements (transcription, DNA breaks, and repair) for CSR and SM. Analysis of germline transcription of Ig C_H exons after activation of CSR in AID-deficient B cells, revealed that AID is not required for the transcription step. Considering DNA repair, AID is also unlikely to function in NHEJ during CSR, because V(D)J recombination, which requires NHEJ, appears to be intact in AID-deficient B cells (11). It has therefore been tempting to speculate that AID (or a RNA transcript edited by AID) functions in DNA cleavage.

In this issue, two manuscripts describe the use of LM-PCR strategies to ask whether AID is required for the DSBs that they previously have found to be associated with SM, and demonstrate, quite surprisingly, that the answer is no (16, 17). Based on the current studies, Papavasiliou and Schatz (16) propose that AID has a post-DNA cleavage function, because they detect DNA breaks by LM-PCR in germinal center B cells from AID-deficient mice, as well as in a B cell line made functionally deficient for AID with a dominant-negative protein. Bross et al. use a similar LM-PCR assay to demonstrate DNA breaks in both germline (unrearranged) and rearranged V gene segments of B cells induced for SM, although SM is preferentially targeted to rearranged V genes (17). Moreover, in the absence of AID, no mutations were found despite an abundance of DNA breaks. Thus, Bross et al. (17) also find that DSBs are generated on Ig variable region genes in cells undergoing somatic mutation in the absence of AID, and conclude that these DSB are not sufficient to induce SM.

With respect to the observed DSBs and the role of AID, Bross et al. provide a more equivocal interpretation than Papavasiliou and Schatz, which includes three possible scenarios: (a) AID is upstream of DSBs in SM, and the DSBs observed in their study are irrelevant to SM; (b) AID is upstream of DSBs in SM, but only a minor proportion of the DSBs seen in wild-type B cells are relevant to the process and these are not above the high background levels seen in AID-deficient B cells; and (c) AID is downstream of the breaks in SM as suggested by Papavasiliou and Schatz. Bross et al. feel that possibility (a) is unlikely based on direct and indirect evidence of others that DSBs are involved in SM (2), and that possibility (c) is unlikely based on evidence that AID may function upstream of DSBs in CSR (see below). Thus, they appear to favor the second possibility, which in actuality is not distinguishable by their studies from possibility (a), in that most (and potentially all) DSBs detected by their assays would be irrelevant to SM. If this possibility were correct, it would raise the further questions of why these DSBs are detected at such high and specific levels, in both wild-type and AID-deficient cells, and whether their previous conclusions that such DSBs are related to SM may need re-evaluation.

The findings of Papavasiliou and Schatz (16) and Bross et al. (17) are particularly intriguing in light of recent work suggesting that AID is required for the DNA breaks associated with CSR. Some form of DNA breaks are clearly intermediates in CSR, as the intervening DNA between recombining S regions is deleted (10). Previous studies provided evidence that DSBs can be detected in S regions by LM-PCR in cells activated for CSR (18), and there is evidence that resolution of these breaks occurs by a process that requires the non homologous end-joining factors (19–22). To address the potential role of AID in CSR-related DSBs, Petersen et al. (23) took advantage of the observation that the histone H2AX becomes phosphorylated within seconds of DSB-inducing DNA damage, and the phosphorylated H2AX proteins (γ-H2AX) can be detected in nuclear foci that likely represent sites of damaged DNA. The formation of γ-H2AX foci is thought to represent a very early event in the response to introduction of DSBs in DNA (24). Petersen et al. found that γ-H2AX foci could be detected at Ig C_H genes in B cells undergoing CSR, but not in B cells from AID-deficient mice (23). In the absence of H2AX, CSR was diminished but still occurred at substantial levels. Based on these data, Petersen et al. reasonably argued that AID likely functions upstream of the DNA modifications that initiate CSR. Examination of the factors responsible for H2AX phosphorylation in CSR, as well as its timing relative to the generation of DNA breaks will certainly shed light on its role in this process. However, one must also consider the possibility that the AID-dependent γ-H2AX foci do not reflect the initial DNA lesion in CSR, but rather are intermediates in the repair process itself. This could occur if resolution of an initial DNA break is resolved via a replication-dependent mechanism, as γ-H2AX foci have been correlated with stalled replication forks (25). While Petersen et al. argued that CSR is performed in G1 (23), there is other evidence that CSR is replication dependent, and that switch region sequences can form secondary structures that could predispose to replicational stress (10).

In their study, Petersen et al. also reported that mutations accumulate in the Sμ region of wild-type cells after stimulation but in the absence of CSR (23). Only background levels of mutations were detected in similarly stimulated AID-deficient B cells. Another report from Nagaoka et al. (26) described similar results and showed that many of these Sμ mutations are reminiscent of those found in SM, occurring at similar hotspot sequence motifs. The Peterson et al. (23) and Nagaoka et al. (26) studies argued that the S re-

gion mutations are markers for the DNA lesions that initiate CSR, because they occur on alleles that had not undergone bona fide CSR (e.g., did not delete the very 3′ of the Sμ core region). They therefore proposed that the absence of the mutations in AID-deficient cells is evidence that AID is required for DNA cleavage in the initiation of CSR. Notably, however, previous studies showed that CSR is accompanied by a very high level of mutations and small deletions in regions flanking CSR junctions (27–29); this was attributed to an error-prone repair process in the resolution stage of CSR. In this regard, it is conceivable that the much lower level of mutations observed by Petersen et al. and Nagoaka et al. might represent an extension of the same process. Thus, while the authors clearly show that such mutations occur in the absence of CSR involving two S regions, they have not ruled out possible contributions of related intra-S region recombination. In this regard, large internal S-region deletions, detectable by Southern blotting, can accompany and precede actual CSR between different S regions and are thought to occur by the same mechanism as bona fide CSR (4). Notably, such deletions occur at frequencies as high as 25–30% in IgM-producing cells activated for CSR and at much higher levels on unswitched alleles in IgG1- and IgG3-producing cells (30–32). Related smaller deletions, undetectable by Southern blotting, also occur. Thus it remains a formal possibility that the S region mutations observed by Petersen et al. and Nagoaka et al. could have been due to error-prone resolution of internal S region recombination events. Thus, more direct DSB assays will be required to draw definitive conclusions about the role of AID in generating CSR-related DSBs (6–9, 16–18, 23).

Taken together, these recent reports imply that while AID is required for DSB formation in CSR, it is dispensable for the SM-associated DSBs detected by Papavasiliou and Schatz, and Bross et al. (16, 17). One explanation for these seemingly disparate findings is that despite the mechanistic similarities between these two processes, AID functions at different steps of the CSR versus SM reactions, e.g., involved in the generation of DSBs in CSR, but in the repair of DSBs in SM. This could be the case if AID edits two different mRNAs, one encoding a factor required for SM and the other for CSR. The requirement for AID in generating DNA breaks during Ig variable region gene conversion has not yet been explored. Could AID function after DNA cleavage in both CSR and SM? As proposed by Papavasiliou and Schatz (16), AID could favor the use of an error-prone DNA repair pathway during SM. In the absence of this AID-dependent pathway, the generation of DNA breaks would be resolved by an error-free mechanism, and mutations would not occur. In the case of CSR, AID could also be involved in a post-cleavage event, if this event is before formation of γ-H2AX foci. This function could also involve recruitment of an error-prone repair pathway, which would introduce mutations around the regions of CSR junctions. In the absence of AID, DNA breaks would be sealed correctly and preclude mutations and recombination. Finally, although the Papavasiliou and Schatz (16) and Bross et al. (17) reports provide strong evidence that AID is dispensable for detection of DSBs in Ig variable regions genes in cells stimulated for SM, it remains formally possible that AID functions upstream of the breaks that initiate SM. In this case, one would have to propose that although most or all DSBs detected in these studies are very specifically correlated with essentially all known aspects of SM, they are nonetheless not directly related to the SM mechanism (16).

One important issue raised by the possibility that AID is required for DNA cleavage in SM, CSR, and gene conversion is how AID-initiated breaks in each process result in different outcomes. It is possible that the breaks are generated during different phases of the cell cycle, when different repair pathways are preferentially employed for DSB repair. Indeed, CSR has been argued to occur during G1 (23), when NHEJ is preferentially active, whereas SM has been argued to occur during G2 (7), when homologous recombination pathways are activated. It may be that SM breaks can be repaired by homologous recombination pathways in G2 because of the presence of a sister chromatid (7), while CSR breaks occur in G1 in the absence of such a template and therefore are resolved by NHEJ. Additionally, SM was not impaired in stimulated B cells in the absence of the NHEJ factor DNA-PKcs, which is required for efficient CSR (19, 22, 33). More generally, the means by which DSBs are resolved may be modulated by factors that preferentially activate one repair pathway over another. Such a model, where tipping the balance of repair factors alters the outcome of DNA breaks, has been recently proposed in a study of gene conversion in the DT40 chicken B cell line (34). This cell line undergoes gene conversion at a high frequency, but when made deficient for the homologous repair factors XRCC2/3, the cell line instead diversified its assembled Ig light chain variable region gene using a SM-like mechanism. If AID functions in a post-cleavage event, it could play a role in altering the balance of factors that are available to repair a break. For example, Papavasiliou and Schatz (16) argue that in the absence of AID, the SM-associated DSBs cannot be repaired by error-prone pathways, and are therefore repaired by error-free pathways.

The factors affecting the outcome of Ig locus DNA breaks may well be generally expressed and found in many cell types. Evidence for this was recently demonstrated in SM studies in plasma cell lines that do not express AID and normally do not undergo SM (35). Expression of AID in these cells allowed SM to occur, indicating that AID is the only factor required for SM that is normally missing in these cells. Remarkably, another recent study showed that AID is the only B cell specific factor required for CSR, because expression of AID in a fibroblast cell line (NIH3T3) was sufficient to cause recombination of a model CSR substrate (36). However, it is clear that within endogenous loci in normal lymphocytes, the factors and processes associated with germline transcription are also required (4), providing two separate levels for control of CSR in activated B cells. The finding that AID can effect CSR in transcribed S re-

gions in nonlymphoid cells raises the intriguing possibility that, regardless of whether AID functions upstream or downstream of DNA breaks, ectopic or dysregulated expression of AID could predispose to tumorigenesis. Specifically, ectopically expressed AID could generate DNA breaks that predispose to translocation, or alternatively, could inactivate specific repair pathways that suppress translocation. Clearly the recent studies examining DNA breaks in CSR and SM in the absence of functional AID have set the groundwork for further investigation of the role of this protein in regulated genetic modifications.

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