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Class-switch recombination: after the dawn of AID

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Abstract

Isotype class switching is central to the humoral immune response. The discovery that mutations in the activation-induced deaminase (AID) gene inhibit class-switch recombination, somatic hypermutation and gene conversion is a major step forward in defining the underlying mechanisms of these gene modification events. The propensity of mutations to occur at dC/dG nucleotides during somatic hypermutation and the homology between AID and cytidine deaminase has resulted in studies demonstrating that AID has the properties of a cytidine-specific mutator and also that elements of the base-excision repair pathway play a central role in class switching and hypermutation. AID is not a promiscuous mutator in the B cell, suggesting that there are specific molecular targeting mechanisms that regulate the accessibility of DNA to AID and differentially regulate class-switch recombination and somatic hypermutation. During class switching, isotype-specific targeting occurs independently of AID and provides another level of specificity to this recombination event.

Introduction

Humoral immunity is dependent on the expression of antibodies with specificity for antigen and specific effector functions. Three types of immunoglobulin (Ig) gene alterations are required to diversify Ig to cover the required range of antigens and produce high-affinity antibody in response to antigen. First, IgV regions are encoded by variable (V), diversity (D) and joining (J) germline gene segments, which are assembled during early B-cell development by the process of V(D)J joining [1]. Second, when mature B cells are stimulated by antigen and the appropriate lymphokines, they switch from IgM expression to produce new isotypes while maintaining the same antigen-binding specificity through a process termed class-switch recombination (CSR; [2,3]). The third genetic alteration that leads to high-affinity Ig production occurs during T-cell dependent antigen responses in which B cells form germinal centers and IgV genes are subject to targeted somatic hypermutation (SHM; [4]). Amongst the three types of Ig gene rearrangements, CSR has been the most enigmatic and refractory to molecular insight.

There are eight heavy chain constant region (C_H) genes each with distinct effector functions in the murine IgH constant region locus, which is organized: 5'-VDJ- C_{μ} - C_{δ} - $C_{\gamma 3}$ - $C_{\gamma 1}$ - $C_{\gamma 2b}$ - $C_{\gamma 2a}$ - C_{ϵ} - C_{α} -3' (Figure 1a). Isotype class switching involves an intrachromosomal deletional rearrangement that focuses on regions of repetitive switch (S) DNA located upstream of each C_H gene (with the exception of C_{δ} ; Figure 1a). During CSR, composite S_{μ} - S_x (where x denotes S_{γ} , S_{α} , S_{ϵ}) junctions are formed on the chromosome and the

intervening genomic material is looped-out and excised as a circle. The recombination breakpoints in these large circles are found to fall largely, but not exclusively, within the S region tandem repeats [5,6]. Work using mitogens and cytokines in primary B-cell cultures indicates that CSR is targeted to specific S regions by the production of germline transcripts (gts; reviewed in [2]). Although CSR is critically dependent on the expression of gts, the function of these transcripts is unknown. It has been speculated that transcription of S regions either changes the chromatin configuration in S DNA making it accessible to factors that mediate CSR or plays a direct role in the CSR mechanism (reviewed in [2,3]). Recent studies indicate that CSR is resolved through a nonhomologous end-joining (NHEJ) pathway ([7–9,10•]; see also Update).

The recent identification of activation-induced deaminase (AID) as a unique regulator of CSR represents a major advance towards more fully understanding the mechanism of this puzzling process. AID expression is restricted to mature activated B cells and was originally detected in a subtractive hybridization screen for genes activated upon induction of CSR in a subclone of the CH12.LX B lymphoma cell line [11]. Exciting new work based on the discovery of AID indicates that there is an unexpected link between CSR and SHM [12]. In the past year, intensive investigation into the role that AID plays in CSR, SHM and gene conversion suggests the outlines of the long-elusive initiating mechanisms of these processes and indicates features unique to CSR.

In this review, I will summarize the newly identified properties of AID and relate them to a potential role in the mechanisms of CSR and SHM. New information regarding isotype-specific targeting of S regions in CSR and its relationship to AID is also presented.

The requirement for AID links CSR and SHM

AID-deficient B cells express slightly elevated IgM in comparison to wild-type (WT) cells, but no other Ig isotypes are produced, even though B cell activation, gt expression and germinal center formation are normal [13]. Mutations in the AID gene lead to deficits in CSR, SHM and gene conversion of IgV genes, suggesting a mechanistic link between these three apparently unrelated processes (Figure 1; [13,14,15•,16•]). The use of SHM or gene conversion for diversification of V genes is species dependent, but in some instances overlapping [17–19]. The chicken uses gene conversion to diversify V genes by incorporation of templated sequence copied from upstream pseudogenes (Figure 1c). Disruption of the AID gene in a chicken B-cell line blocks gene conversion of V genes and the block can be reversed by AID cDNA [15•,16•]. In mice and humans, V genes acquire untemplated single base changes through SHM (Figure 1b). Ectopic expression of AID in fibroblasts or hybridoma cell lines stimulates mutation of transfected green fluorescent protein (GFP) genes and endogenous IgV genes, respectively [20•,21•]. Similarly, ectopic expression of AID in mouse fibroblasts is sufficient to stimulate CSR of integrated switch substrates, thereby demonstrating the dependence of CSR on AID [22•]. Together, these findings indicate that AID is the only B-cell specific factor required to support CSR, SHM and gene conversion in the context of non-B cells.

The AID protein sequence is homologous to the mammalian RNA-editing deaminase, APOBEC-1 which is the catalytic component of the editsome for apolipoprotein-B mRNA [11,23]. On the basis of its homology to APOBEC-1, and its ability to deaminate deoxycytidine *in vitro* [11], it is possible that AID has an RNA-editing function that deaminates a specific cytosine residue, thus altering the function of an encoded protein(s) involved with CSR, SHM and gene conversion. Alternatively, AID might directly target the relevant RNA or DNA sequences for recombination or mutation.

The DNA cytidine deaminase model for AID action in CSR, SHM and gene conversion

The possibility that AID might act directly on DNA to deaminate dC residues was suggested by several studies. In SHM, transition mutations occur more frequently *in vivo* than is predicted if mutations occur randomly [24]. A higher mutation frequency of G–C base pairs (bps) located in hotspot motifs is also observed [25]. Ectopic expression of AID in fibroblasts or hybridoma cells induced mutations at dC/dG bps, and most of these mutations were transitions [20•,21•]. The observation that mutations focus at dC/dG bps prompted Neuberger and co-workers [26••] to test the hypothesis that AID functions as a cytidine deaminase resulting in the formation of uracil residues. They reasoned that if AID directly deaminates dC then expression of AID in *Escherichia coli* would give rise to a mutator phenotype that targets dC/dG bps, whereas hypermutation in *E. coli* would be an unlikely outcome if the primary function of AID is to edit mRNAs [26••]. In support of this model, ectopic expression of AID in *E. coli* led to increased mutation in several target genes, and a shift towards dC→dT and dG→dA transitions [26••]. However, the incidence of AID-induced mutation in *E. coli* was very low and it remains unclear whether the DNA cytidine deaminase activity observed is unique to AID or whether other RNA editing enzymes are also capable of deaminating dC in DNA (see also Update).

The Neuberger laboratory [26••] hypothesized that if the formation of dU/dG mismatches resulting from deamination of dC residues was a crucial step in CSR and SHM then a block in the base-excision repair (BER) pathway might alter the outcome of these processes (Figure 2a). In the BER pathway uracil is removed by uracil-DNA glycosylase (UNG; [27]). This is followed by cleavage of the sugar-phosphate chain by apurinic apyrimidinic endonuclease (APE), excision of the abasic residue and local DNA synthesis and ligation. Perturbation of the BER pathway by mutation of UNG would force the rectification of mismatches through DNA replication or mismatch repair (MMR) proteins and might impact on the types of mutations found in SHM (Figure 2a; summarized in [28]). Petersen-Mahrt and co-workers [26••] show that, in *E. coli*, AID-triggered mutation is synergistically enhanced by UNG deficiency, supporting the notion that deaminated dC residues in DNA are generated by AID. Di Noia and Neuberger [29••] have taken this analysis a step further and have studied chicken DT40 cells for the effect of UNG deficiency on SHM. DT40 cells that lack the DNA-repair gene XRCC2 almost exclusively undergo transversion-favoured dC/dG hypermutation [30,31•]. The transversions are proposed to arise from DNA replication over sites of base loss produced by the excision function of UNG (Figure 2a). A key prediction of the deamination model is that UNG deficiency will lead to DNA

replication over the dU lesion resulting in transitions. In DT40 XRCC2^{-/-} cells, inhibition of UNG by ectopic expression of the uracil-DNA glycosylase inhibitor (Ugi) protein leads to a shift from IgV gene transversion-dominant to transition-dominant mutations, as predicted [29••].

In the mouse there are at least four uracil excision enzymes and each might be involved in resolving dU residues in SHM and CSR [32–35]. Rada and co-workers [36••] examined the contribution of UNG to SHM and CSR in UNG-deficient mice and found UNG to be the major mouse DNA glycosylase involved in processing dU residues in SHM [36••]. Furthermore, analysis of CSR in UNG-deficient LPS-activated B cells demonstrated that CSR was substantially, although not completely, inhibited, thereby implicating the BER pathway in the CSR reaction. Rada and co-workers [36••] postulate that residual CSR is caused by the use of other dU glycosylases or engagement of the MMR pathway. CSR frequency is partially reduced in mice deficient for MMR proteins [37,38]. These findings strongly support the involvement of uracil repair through BER in both CSR and SHM, and imply that AID functions to initiate these processes by dC deamination. An important question arising from these findings is: ‘How does the DNA lesion arising from dC deamination avoid completing repair through BER and instead is recruited into the CSR or SHM pathway?’

DNA double-strand breaks probably initiate CSR

The production of excision circles as a direct by-product of CSR suggests a concerted mechanism in which DNA is broken and rejoined in the recombination reaction. Blunt and staggered double-strand breaks (DSBs) have both been implicated as initiating events in CSR [39,40]; CSR is initiated by DSBs specific to the S regions and completed by rejoining the ends to form S–S recombinant junctions. However, it is unclear whether AID generates DSBs (directly or indirectly) or whether this activity represents a general recombinase that, in combination with gts, acts at specific S regions or whether there are isotype-specific docking proteins as well.

DNA DSBs stimulate the phosphorylation of histone H2AX, a process that is critical in recruiting repair proteins to DSBs [41]. B cells isolated from H2AX-deficient mice were activated with LPS and IL-4 and showed a significant, although incomplete, reduction of $\mu \rightarrow \gamma 1$ switching, indicating that H2AX is an important facilitator of efficient CSR [42••]. In B cells activated to switch, co-localization of phosphorylated H2AX (γ -H2AX) with Nijmegen breakage syndrome protein (Nbs1; another DNA repair protein) in nuclear repair foci was observed [42••]. Recruitment of the IgH locus to the Nbs1 and γ -H2AX-associated nuclear foci occurred in AID-positive but not in AID-negative B cells. Petersen and co-workers [42••] concluded that AID functions upstream of H2AX phosphorylation and might be responsible for the generation of DNA breaks that initiate CSR.

S–S recombination junctions are frequently flanked by deletions, duplications and mutations that were thought to accumulate through an error-prone repair pathway [43,44]. Recently, studies have shown that a high incidence of mutations occurs in the regions flanking S μ in WT LPS B cells, but are absent in AID-deficient B cells, suggesting that the generation of

mutations is integral to the process of CSR [42••,45•,46]. On the basis of these observations it is possible to conclude that AID produces S region-specific DNA lesions that lead to the mutations and DSBs that initiate CSR.

The AID deamination model, as formulated by Neuberger and co-workers [26••], predicts that staggered DSBs could emerge following deamination of closely spaced dC residues located on opposing strands in the S region (Figure 2b). In this hypothesis, the resulting dU residues are removed by UNG to form abasic sites with the subsequent incision of the phosphodiester backbone by APE, creating staggered DSBs (Figure 3). Alternatively, removal of dU/dG mismatches by MMR proteins could produce short single-stranded gaps on complementary strands of DNA which could also form DSBs. Blunt DSBs can be generated by ‘polishing’ the staggered ends with exonuclease. In this context it is interesting that a B-cell associated 3′→5′ exonuclease has been detected [47]. To determine the role of AID in generating DNA breaks, a direct analysis of DSB formation in S regions of AID WT and knockout B cells activated to switch is required. In CSR, mutations in S DNA are probably a by-product of AID-induced dU lesions. It is notable in this regard that intra-Sμ region deletions are detectable in AID^{-/-} B cells, albeit at much reduced frequency, implying that a pathway for CSR might exist in which DSBs in S regions are generated independently of AID [46].

DNA double-strand breaks in SHM

Several laboratories have investigated the involvement of DNA breaks as the initiating step in SHM (reviewed in [48]). DSB formation in V genes was found to be dependent on transcription and focused at mutational hotspot motifs. DSBs appeared during G2 of the cell cycle, preferentially in cells undergoing SHM. Intriguingly, DSB formation was independent of AID expression [49,50]. Recent studies indicate that DSBs are also found in germline V genes, which are not targets for hypermutation, thereby questioning the relationship of DSBs to SHM [49]. Inducible mutations associated with SHM in the BL2 (Burkitt’s lymphoma) cell line were clearly shown to occur during G1 of the cell cycle [51•]. These results indicate that DSBs found in G2 of the cell cycle are unlikely to be the initiating event in the hypermutating cell line. The precise relationship of DSBs in V genes to SHM is currently unclear.

Isotype-specific targeting of S regions in CSR

The existence of S-region specific recombinases has been suggested on the basis of the unique character of each S region [44]. The degeneracy of S DNA and the absence of consensus recombination signal sequences have led some investigators to suggest that the specificity of the CSR process is achieved exclusively through production of gts and a single recombinase (which could be AID; reviewed in [2,3]). However, in addition to gts and AID there are other *trans*-acting factors that contribute to CSR. The Snapper laboratory [52] defined several B-cell activation protocols and found that some of these induced gts for several isotypes, but supported switching to only a subset of them [52]. Moreover, B cells lacking specific transcription factors maintain gt expression but switch only particular isotypes [53–55]. These findings do not support the minimalist notion that gt expression and

a single recombinase is responsible for CSR because in this model all loci that express gts should switch.

Shanmugam and colleagues [56] sought to test the proposition that isotype-specific *trans*-acting factors contribute to CSR using extrachromosomal switch substrates. The transfection of isotype-specific S plasmids into a panel of mature B-cell lines with well-characterized switching phenotypes and normal mitogen-activated splenic B cells, demonstrates the existence of distinct switching activities that mediate $\mu \rightarrow \gamma 3$, $\mu \rightarrow \gamma 1$, $\mu \rightarrow \epsilon$ and $\mu \rightarrow \alpha$ CSR [56,57•]. Recently, plasmid recombination was found to be highly dependent on the expression of AID, demonstrating that switching on these plasmids reflects physiological CSR (AL Kenter, H Zhang, unpublished data). In light of new evidence that AID directly deaminates dC residues in DNA, it is tempting to speculate that the isotype-specific *trans*-acting factors are docking proteins that recruit AID to S regions. However, it is equally possible that isotype-specific *trans*-acting factors are suppressors of CSR that perhaps preclude AID interactions with particular S regions and are themselves repressed in response to particular combinations of CSR inducers. Alternatively, the *trans*-acting factors may be involved in recruiting the recombination machinery to the broken S DNA ends to facilitate synapsis and generation of composite S–S junctions. Several DNA-binding proteins have been detected that are specific for S DNA, but evidence has not yet been presented to directly link these proteins to the CSR reaction (reviewed in [2]).

A CSR versus SHM decision fork: targeting S regions or V genes

Studies have clearly shown that the expression of gts and V genes is required for, and proportional to, the frequency of CSR and SHM, respectively [58,59]. Using this criteria, both CSR and SHM should occur in cells that produce gts, transcribe the rearranged V gene and express AID. However, LPS-activated splenic B cells transcribe gts and the rearranged V genes, but only undergo CSR and not SHM [60]. Therefore, CSR and SHM are differentially regulated in B cells. Similarly, CSR and SHM are independently and differentially induced in the CL-01 (Burkitt's lymphoma) cell line [61].

AID is a promiscuous mutator when ectopically expressed in *E. coli* [26••]. In mouse fibroblasts, the P1–5 hybridoma cell line and in CHO cells, AID indiscriminately mutates other ectopically expressed genes, including itself [20•,62]. The mutation of transgenic AID and V genes and endogenous V genes (but not endogenous bcl6 or c-myc) is also evident in B cells that express ectopic AID [21•,62]. These studies demonstrate that DNA context is a major accessibility determinant for AID. How are mutations targeted to the expressed V gene as opposed to the nearby germline V genes and S regions during SHM and, reciprocally, to S DNA and not to V genes during CSR? One attractive explanation is that *cis*-regulatory elements, including promoters and enhancers, control the differential targeting of AID to V and S regions by regulating substrate accessibility.

Although DNA context is clearly important for determining accessibility to AID, *trans*-acting factors may also play a role in directing AID to particular sequences. The mature B-cell lines M12 and A20 constitutively express AID but are unable to support CSR on extrachromosomal switch plasmid substrates [57•]. These cell lines can be induced to initiate

CSR on the plasmid substrates following stimulation with combinations of CD40L and other inducers. The same conditions also stimulate CSR at the endogenous S regions in M12 cells, suggesting that another, unknown, regulator(s) directs efficient CSR in the presence of AID. An important question raised by these studies is: 'How is AID targeting modulated in B cells to repress or induce the AID mediated mutation?'

Conclusions and future challenges

The discovery of AID and the recognition that CSR, SHM and gene conversion are mechanistically linked represents a change in our understanding of each of these processes. The deamination model for the introduction of DNA lesions, resulting in the initiation of CSR, SHM and gene conversion, is a powerful hypothesis that elegantly outlines a role for AID in each of these processes. The involvement of the BER pathway in both CSR and SHM strongly supports the deamination model. It is clear that AID is a global mutator when ectopically expressed. Direct biochemical evaluation of AID's contribution to lesion production will be a focus of research in the near future.

There are several major new questions looming on the CSR/SHM horizon. The differential induction of CSR and SHM in B cells indicates that region-specific targeting of AID to S DNA or expressed V genes must occur. The challenge is to define both the *cis*- and *trans*-regulatory targeting elements governing the decision to switch or hypermutate. When lesions are introduced and UNG is invoked, the recombination intermediates avoid complete repair by BER and funnel instead through the mutation-generating or recombination machinery. The characterization of factors involved in the decision to repair or mutate/recombine will probably add important new insights to our current understanding. *Trans*-acting isotype-specific factors, distinct from AID, have been implicated in CSR. Identification of the protein components of these activities, definition of their recognition motifs and characterization of their role in the recombination machinery will provide new perspectives on the specificity of CSR. Overall, it has been a good year for CSR and SHM.

Update

The Neuberger laboratory has recently reported that APO-BEC1, APOBEC3C, APOBEC3G and AID all have cytidine deaminase activity, but with distinct local target specificity. This questions how AID achieves specificity in B cells and avoids becoming a generalized mutator [63•].

Complementation of DNA-PKcs-deficient B cells (in which some DNA-PKcs protein is expressed but does not display the kinase activity) with rearranged H- and L-chain genes shows intact CSR to all isotypes [64•]. These findings suggest that the DNA-PKcs protein has a function in CSR separate from its kinase activity.

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Abbreviations

AID	activation-induced deaminase
APE	apurinic apyrimidinic endonuclease
BER	base-excision repair
bps	base pairs
C_H	heavy chain constant region
CSR	class-switch recombination
D	diversity gene segment
DSBs	double-strand breaks
gt	germline transcript
Ig	immunoglobulin
IL	interleukin
J	joining gene segment
MMR	mismatch repair
Nbs1	Nijmegen breakage syndrome protein
NHEJ	nonhomologous end joining
S	switch
SHM	somatic hypermutation
UNG	uracil-DNA glycosylase
V	variable gene segment

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Figure 1.

Class switch recombination, somatic hypermutation and gene conversion. **(a)** CSR in the IgH gene. The IgV gene is assembled from the V, D and J gene segments in early B-cell development. CSR occurs in mature B cells by looping-out and deletion of intervening genetic material. At the top, a partial schematic map of the IgH locus before CSR is shown. IgM and IgD are transcribed from the same V_H promoter and are expressed by alternative RNA processing. A germline transcript is shown which must be expressed *in cis* to target switching. In the middle diagram, the intrachromosomal deletion that results in the switch

from IgM to IgG1 production is shown. The arrows indicate AID-initiated DSBs in S regions. At the bottom, the IgH chromosome following CSR is shown. (b) Somatic hypermutation (SHM) and (c) gene conversion are processes that require gene transcription and AID. **(b)** During SHM, mutations are restricted to the region close to the V_H promoter and spread through the assembled V gene but are not evident in the C_H gene. **(c)** During gene conversion, information located in an upstream pseudogene is copied into the expressed V region gene. Gene conversion is the dominant mechanism for diversification of V genes in chicken and rabbits.

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Figure 2.

The possible outcomes of AID cytidine deaminase activity on IgH genes. **(a)** There are several DNA repair options for the AID-generated dU/dG mismatch; DNA replication, base excision by UNG followed by replication, and MMR followed by error-prone DNA synthesis, all of which generate mutations. Replication of the DNA strand harboring the dU residue will produce a dC→dT (and a dG→dA) transition. If, instead, DNA synthesis occurred over the abasic site then both transitions and transversions would be generated. MMR will recognize and excise the area around the dU/dG mismatch to produce a gap that can be repaired by error-prone polymerases. **(b)** CSR and gene conversion are likely to be initiated by DSBs. When two closely spaced dC residues are deaminated on opposite strands, UNG and apurinic apyrimidinic endonuclease (APE) or MMR activities could generate staggered DSBs. APE functions to incise the sugar-phosphate backbone at the abasic site to produce a single-strand nick in the DNA. Two closely placed nicks on opposing DNA strands will give rise to a staggered DSB.

Figure 3.

A schematic summary of how the base-excision repair and mismatch repair pathways produce double strand breaks. AID (either directly or indirectly) deaminates cytosine residues (C) and transforms them into uracil residues (U). The guanosine (G)/U mismatches are recognized and excised along either one strand or the other, by the MMR system, to form small gaps in the DNA. If the gaps are closely spaced a staggered DSB can form. If the MMR-generated gaps do not overlap they will be repaired by error-prone polymerases and thereby cause mutations. Alternatively, the uracil residues in the G/U mismatches trigger the BER pathway and are then recognized by UNG and excised. The APE cleaves at the closely spaced abasic sites to form staggered DSBs. Blunt DSBs form if the staggered ends are

polished by exonuclease. Mutations are associated with recombinant S/S junctions when error-prone polymerase(s) is involved in resecting S DNA during the recombination reaction.

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