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Review

AID in antibody perfection

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Abstract. Expressed immunoglobulin (Ig) genes undergo alterations in sequence and genomic structure in order to optimize antibody function. A single B cell-specific factor, activation-induced deaminase (AID), initiates these changes by deamination of cytosine to uracil. Uracil in DNA is encountered commonly, and conserved pathways are responsible for its faithful repair. However, at the Ig loci of B cells, AID-initiated damage is processed to produce three distinct outcomes: somatic hypermutation, class switch recombination and gene conversion. This review focuses on the role of AID in Ig gene diversification, emphasizing how AID functions within the mechanism of the Ig gene diversification pathway; and highlights open questions for future research, particularly the most provocative current question: what makes a gene a target for AID-initiated mutagenesis?

Keywords. Activation-induced deaminase, immunoglobulin, somatic hypermutation, class switch recombination, gene conversion.

Introduction

The immune system provides a vital defense against infection by pathogenic microorganisms, and antibodies are central to the immune response. Antibodies are produced by B cells, which derive from hematopoietic precursors in the human bone marrow, or in the bursa of Fabricius in the chick. B cell development and activation is regulated by T cells, which populate the thymus. To be useful, both B and T cells must express a diverse receptor repertoire capable of recognizing many different antigens. In T cells this is achieved exclusively by combinatorial joining of V, (D) and J segments in early cell development. In contrast, recombination of V, (D) and J segments is a necessary step in B cell development, but this is not the only stage at which repertoire diversification can occur. B cells have additional sophisticated mechanisms to further diversify immunoglobulin (Ig) sequence and structure.

Ig gene diversification refers specifically to the mutagenic processes that alter the sequence and structure of functional Ig genes, which are already producing antibody molecules. In some cases, such as chicken or sheep, Ig gene diversification can expand the pre-immune repertoire prior to encounter with antigen. In humans or mice, Ig gene diversification is critical for producing antibody molecules capable of high-affinity recognition of specific antigens, and it provides a dynamic response against pathogens, which are themselves constantly evolving.

Ig gene diversification in response to challenge with antigen occurs in specialized secondary lymphoid organs, such as spleen and lymph nodes. Antigen-activated B cells first expand in the periarteriolar lymphoid sheaths (PALS). Subsequently, B cells populate specialized microenvironments, called ger-

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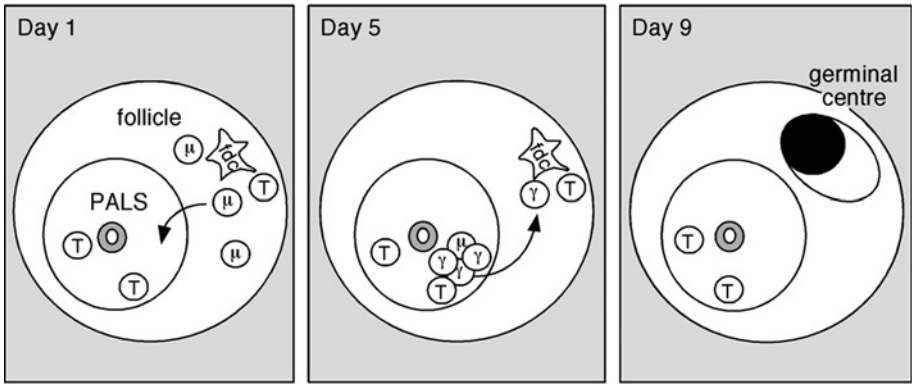

Figure 1. Activation of B lymphocytes. In secondary lymphoid organs, B cells are activated by antigen and contact T cells in the PALS (ring designates central arteriole). B cells then migrate to the follicle and form germinal centers. B and T lymphocytes and follicular dendritic cells are indicated.

minal centers, where they come into contact with T cells and follicular dendritic cells displaying antigen on the cell surface (Figure 1). In the germinal center, diversification is coupled with selection for B cells expressing high affinity Ig molecules.

Three mutagenic outcomes of Ig gene diversification

Three processes come under the umbrella of Ig gene diversification, each characterized by a distinctive mutagenic signature (Figure 2) [reviewed in ref. 1].

1) Somatic hypermutation introduces point mutations into rearranged and expressed Ig V regions, deleting existing DNA sequence. In antigen-activated B cells, hypermutation is coupled with clonal selection for cells expressing high-affinity antigen receptors, and thus increases the affinity and specificity of the immune response. Hypermutation also provides a dynamic response to pathogenic microorganisms, which are themselves constantly mutating.

2) Class switch recombination joins the expressed V region to a new downstream constant (C) region, in a process of regulated DNA deletion. Different C regions are specialized for different modes of antigen clearance, so by literally switching one C region for another, switch recombination optimizes the mode of clearance of antigen from the body. Switch recombi-

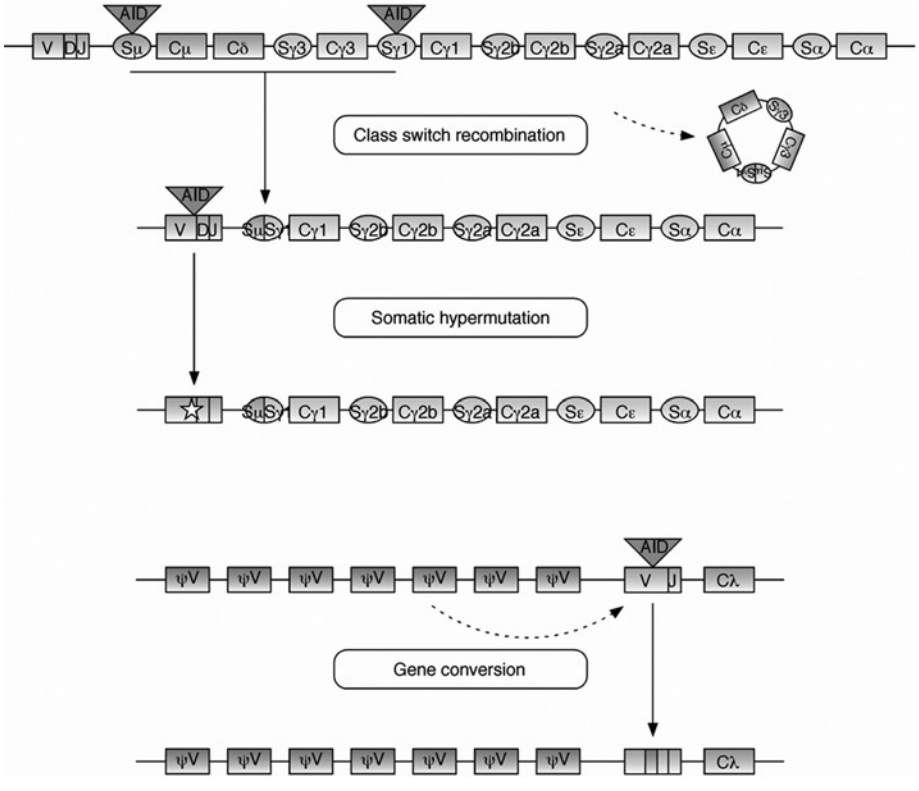

Figure 2. Immunoglobulin gene diversification: class switch recombination; somatic hypermutation; and gene conversion. Class switch recombination causes deletion of DNA to join the expressed V region to a new C region. Somatic hypermutation introduces point mutations in the V region of the expressed Ig gene. Gene conversion introduces templated mutations into the V region. All three processes are initiated by AID (triangle). V, variable region; S, switch region; C, constant region. See text for details.

nation does not affect V regions, but creates junctions within switch (S) regions, located upstream of each C region. G-rich sequences in S regions that are 2–10 kb in length participate in switch recombination.

3) Gene conversion (templated mutagenesis) transfers tracts of sequence from a family of nonfunctional donor pseudo-V regions to the rearranged and expressed V region. Gene conversion is the primary mechanism for diversifying the pre-immune repertoire of chickens and other fowl, as these creatures have only a limited number of functional V regions.

These three processes of Ig gene diversification were long thought to depend on distinct mutagenic mechanisms, largely because the products of mutagenesis are distinct. Hypermutation produces non-templated, single-base changes; switch recombination causes extensive deletion of chromosomal DNA, creating new junctions within G-rich, repetitive regions; and gene conversion inserts tracts of templated mutations copied from homeologous and nonfunctional donor genes. But all three processes have proved to share significant mechanistic features, most notably dependence upon the single B cell-specific enzyme, activation-induced deaminase (AID) [2–5].

AID was discovered only 6 years ago, but since then there has been extraordinary progress in understanding the detailed mechanisms of all three processes of Ig gene diversification. This review begins with some of the history of that discovery, discusses how AID functions within the mechanism of Ig gene diversification and highlights open questions for future research.

AID is an essential enzyme for Ig gene diversification

AID was first identified by Honjo and colleagues in subtractive hybridization experiments designed to enrich for mRNAs displaying increased expression upon activation of switch recombination in a cultured murine B cell line [6]. Genetics provided the proof that AID is key to Ig gene diversification. Honjo's laboratory demonstrated that targeted deletion of the gene encoding AID in mice prevented both class switch recombination and somatic hypermutation [4]. In a back-to-paper, Durandy's laboratory showed that deficiency in AID is responsible for a human immunodeficiency, HIGM2, characterized by the absence of switch recombination and somatic hypermutation, and overproduction of IgM antibodies [5]. Shortly thereafter, AID was shown to be essential for gene conversion in chicken B cells [2, 3].

The possibility that somatic hypermutation, class switch recombination and gene conversion might all share at least some aspects of a common mechanism had been anticipated. That templated and nontemplated mutagenesis represented alternative outcomes in repair of a DNA break was first pointed out over a decade ago [7]. This received experimental support with the demonstration that in chicken B cells lacking factors essential for recombinational repair, the balance of mutagenesis is shifted from gene conversion (templated mutation) to point mutations (non-templated mutagenesis) [8].

AID deaminates C to U to promote mutagenesis

The *AID* gene (officially known as the *AICDA* gene) encodes a 28-kDa polypeptide closely related to APOBEC1, the first mammalian RNA-editing activity to be described [9]. This relationship was immediately evident when AID was first cloned and sequenced, and prompted experiments which showed that recombinant protein has cytidine deaminase activity [6]. APOBEC1 deaminates C to U at a single nucleotide in the mRNA encoding apolipoprotein B101, generating a premature stop codon which results in synthesis of a truncated polypeptide of altered function. Homology to APOBEC1 led to extensive and prolonged speculation that the target of AID was a specific mRNA which must be edited to promote Ig gene diversification [10]. If such a target exists, it has yet to be found. A variety of evidence supports the view that AID deaminates DNA at transcribed Ig genes.

The first compelling evidence that AID deaminates DNA to promote mutagenesis came from the Neuberger laboratory. Uracil in DNA is normally excised by uracil DNA glycosylase (UNG), leaving an abasic site for further repair. Neuberger and colleagues reasoned that if AID deaminates DNA directly and uracil is a key intermediate in the mutagenic pathways, then Ig gene diversification should be affected by the absence of UNG activity. One critical test was to ask if AID promoted mutagenesis in *Escherichia coli*. AID was stably expressed in *E. coli*, and mutagenesis of reporter sites was assayed in ung^+ and ung^- backgrounds [11]. Not only did AID expression stimulate mutagenesis, there was increased mutagenesis in ung^- cells. This result was a turning point in thinking about the mechanism of mutagenesis, not only because involvement of UNG provided evidence of the importance of the DNA deaminase activity of AID, but also because it was nearly impossible to argue that the target of AID was an mRNA that was conserved from *E. coli* to mammalian B cells. Shortly thereafter, UNG was shown to be critical for Ig gene diversification [12]. In $Ung^{-/-}$ mice, levels of switch recombination were reduced and the spectrum of hypermutation altered [13].

Figure 3. Defined domains and motifs in the AID polypeptide. Residues that may be phosphorylated are indicated. The C-terminal nuclear export signal is shown (NES); and the N-terminal nuclear localization signal (NLS) is within parentheses to highlight the fact that it has not been shown to be necessary to transport AID into the nucleus.

The importance of UNG for Ig gene diversification in mammals was confirmed by the demonstration that the absence of active UNG results in a human immunodeficiency syndrome [14]. Like AID deficiency, UNG deficiency is characterized by profoundly impaired switch recombination and an altered hypermutation spectrum. Interestingly, human UNG deficiency was first discovered as an immunodeficiency, and affected individuals do not display the predisposition to malignancy usually typical of deficient DNA repair [15].

AID is a conserved protein

The AID polypeptide is highly conserved in organisms ranging from fishes to humans [16,17]. AID contains a Zn^{2+} binding cytosine deaminase motif near the N terminus and a noncatalytic C-terminal domain [18] (Figure 3). As might be expected, mutations within the catalytic domain that ablate the deaminase activity of AID completely block Ig gene diversification [19]. Unexpectedly, mutations within the C terminus abolish the ability of AID to promote class switch recombination, but not somatic hypermutation [19-21]. Conversely, mutations in the N terminus impair somatic hypermutation but not class switch recombination [22]. This separation of functions by deletion mutagenesis has been proposed to reflect a requirement for each domain to recruit factors necessary for downstream steps in diversification. Support for this model comes from evidence that the C terminus of AID interacts with DNA-PKcs [23], a component of the nonhomologous end-joining pathway that may function in class switch recombination [24]. A hinge domain connects the deaminase motif to the C terminus (Figure 3). Crystal structures of cytidine deaminases from *E. coli* and *Saccharomyces cerevisiae* provide the best current models for the binding-fold and active-site architecture of AID [18, 25]. Three dimensional modeling has predicted that AID forms a homodimer, with each subunit contributing to formation of a binding site for single-stranded DNA. Experimental evidence for dimerization has been obtained by immunoprecipitation [19, 26] and mutation of AID [27]. Self-association is a common feature among cytidine deaminases, and this may be important for interaction with specific cofactors or the substrate recognition required for AID to initiate Ig gene diversification [18, 27].

AID deaminates ssDNA at WRC hotspots

Biochemical analysis of purified recombinant AID has provided detailed understanding of how function *in vitro* correlates with features of hypermutated Ig genes [reviewed in ref. 28]. Before the discovery of AID, extensive sequence analysis had been carried out on hypermutated V regions, and the sequence motif WRCY (W = A or T, R = purine, Y = pyrimidine) was found to be a hotspot for hypermutation [29–31]. In the absence of a mechanistic explanation, hypermutation hotspots were initially interpreted to reflect selection for high-affinity antibodies. However, analysis of mutations produced in the absence of selection clearly demonstrated that hotspots reflect an intrinsic property of the mutational mechanism [32, 33]. The explanation for these hotspots became apparent upon sequencing DNA deaminated by purified recombinant AID *in vitro*: the enzyme was shown to preferentially deaminate cytosines in WRC motifs [34, 35].

AID action *in vitro* is clearly processive [35]. Processivity is less evident in genes which diversify in activated B cells, but is evident in the footprint of C to U deamination, preserved as C to T transition mutations in mutant backgrounds in which no diversification can occur [36]. Downstream steps in mutagenesis may inhibit progression of AID along the DNA; or, alternatively, clustered uracils may be more efficient targets for faithful repair.

AID is targeted to transcribed Ig genes

Prior to the discovery of AID, extensive analysis of diversification of endogenous Ig genes and engineered transgenes had established that transcription is prerequisite to diversification; and, strikingly, that rates of hypermutation are proportional to the rate of transcription [37–39]. Coupling of diversification to

transcription was explained in part by evidence that AID preferentially deaminates C to U in single-stranded and not double-stranded DNA [26, 40-43]. The preference of AID for single-stranded substrates immediately suggested that passage of the transcription apparatus is necessary to transiently denature DNA and create a single-stranded substrate for AID. Transcription may also play another role in targeting AID. Chromatin immunoprecipitation has shown that AID may associate with transcribed Ig genes, via a direct interaction of AID with the transcription apparatus [44]. The details of such an interaction remain to be explored.

How does AID access the template DNA strand?

Thus far it has been difficult to correlate the strong preference of AID for single-stranded substrates *in vitro* with considerable evidence showing that there is no strand bias *in vivo* [45]: i.e., comparable numbers of mutations accumulate at Cs (or WRC motifs) in both strands. In addition, double-strand breaks are substrates of the nonhomologous end-joining pathway, which promotes class switch recombination [46], but if AID attack is restricted to the nontemplate strand, then how are breaks in the template strand created? A single-strand break could be converted to a double-strand break upon replication; or, alternatively, AID could attack both strands. The latter possibility has received support from evidence that in $Ung^{-/-}Msh2^{-/-}$ mice, which are unable to repair U/G mismatches introduced by AID, there is an equivalent level of mutagenesis of C to U on both DNA strands [36].
Several possible mechanisms have been identified that may provide AID access to the template strand. It has been suggested that local structural variation, created for example by passage of the transcription apparatus, may provide single-stranded DNA targets for AID on both strands [47, 48]. Interactions of AID with replication protein A (RPA) have been shown to promote access to the template DNA strand [49]. Post-translational modifications regulate the activity of AID and may promote activity on double-stranded substrates.

Life cycle of AID

AID is specifically expressed in germinal center B cells [50]. Germinal centers are specialized physiological microenvironments, where somatic hypermutation and switch recombination occur, and where diversified B cells undergo antigen-driven selection [51] (Figure 1). Surprisingly for an enzyme which modifies DNA sequence, AID localizes predominantly to the cytoplasm [52] (Figure 4). Moreover, while lacking a clear nuclear localization signal, AID contains a consensus motif that promotes nuclear export via the CRM1-dependent pathway [53]. AID is small enough (28 kDa) to enter the nucleus by diffusion, explaining the lack of a nuclear localization signal; but the significance of the requirement for a functional nuclear export signal is not understood.
Phosphorylation of S38 is essential for AID interaction with RPA (Figure 4), and this association modulates AID activity on transcribed substrates *in vitro* and affects levels of class switch recombination [49, 54, 55]. The residue S38 is a target for phosphorylation by protein kinase A (PKA), and inhibition of PKA blocks class switch recombination [55]. Phosphorylation of AID significantly increases its deaminase activity on duplex DNA, although the phosphorylated enzyme still retains its preference for single-stranded substrates [54, 56].

The mechanism of AID-initiated Ig gene diversification

The uracil produced upon AID-initiated deamination has three possible fates, each of which contributes differently to Ig gene diversification. It can undergo replication and become fixed as a transition mutation; or it can be processed by pathways dependent upon uracil excision or mismatch recognition. These latter pathways are redundant and complementary pathways for hypermutation and class switch recombination, as deficiency of either *Ung* or *Msh2* alone is not sufficient to completely abolish either process in mice [57].

Replication

Replication of a U/G mismatch results in a C-T transition mutation, and can contribute to point mutagenesis. It cannot support switch recombination or gene conversion, both of which depend on a DNA break. Replication could also result in other mutations if alterations in nucleotide pools promoted misincorporation opposite U, at abasic sites, or in the course of repair [58]. However, there is no evidence thus far of nucleotide pool imbalances in activated B cells.

UNG-dependent diversification

Uracil excision by UNG [12–14, 59, 60] or another uracil DNA glycosylase, SMUG1 [61] generates an abasic (AP) site (Figure 5). The conserved and ubiquitous factor MRE11/RAD50/NBS1 (MRN) cleaves DNA at AP sites generated by successive action of AID and UNG, using the highly conserved

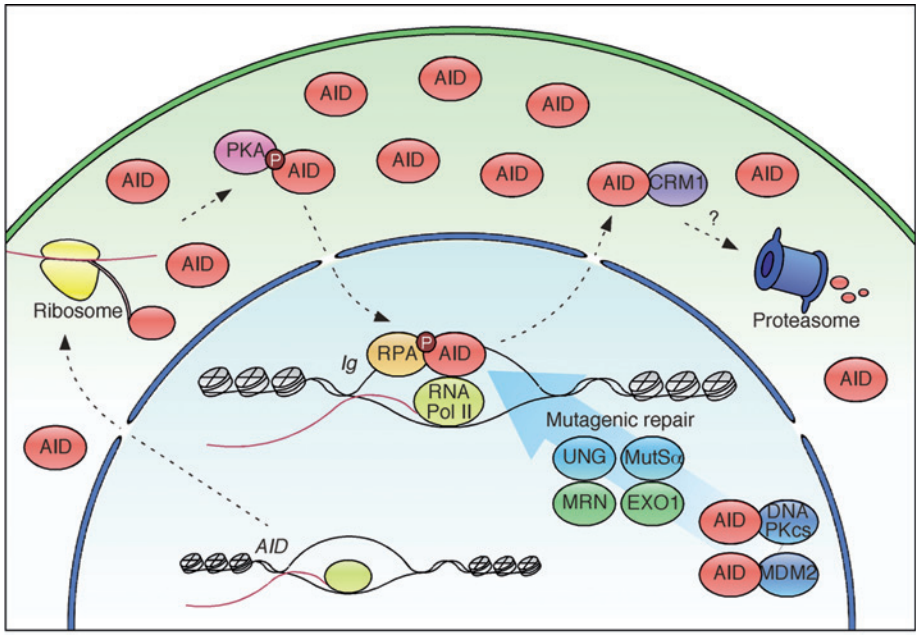

Figure 4. Life cycle of AID. AID is expressed in response to antigen activation. AID protein predominantly localizes in the cytoplasm (light green) and diffuses or is actively transported into the nucleus (light blue). Nuclear AID is shown to be phosphorylated, an event mediated by protein kinase A (PKA). AID targeting to the Ig loci depends upon binding partners including RPA and RNA pol II; and also on chromatin status. AID has also been shown to be excluded from the nucleus through a CRM1-mediated pathway, which may promote its active degradation.

and previously undiscovered MRE11 AP lyase activity to produce a single-strand break (SSB) [62, 63]. This SSB could promote either gene conversion or switch recombination. Uracil excision alone is sufficient to support gene conversion, as gene conversion is essentially abolished in chicken B cells lacking UNG activity [59, 60].

Error-prone DNA synthesis opposite the AP site or at the break, by any of several mutagenic polymerases, could create a broad mutagenic spectrum in hypermutation. Error-prone polymerases currently implicated in UNG-mediated mutagenesis are Polθ and Rev1. Deficiency of Rev1, a Y-family polymerase, causes a near absence of transversions at C/G residues [64, 65]. Polθ is a very large A-family polymerase. Mice deficient in polθ show a reduction in hypermutation levels and an altered hypermutation spectrum [66, 67].

MutSα-dependent diversification

MutSα (the MSH2/MSH6 heterodimer) binds to U/G mismatches, initial products of AID deamination [68, 69], in an interaction analogous to mismatch recognition that initiates canonical mismatch repair (Figure 5). Deficiencies in MSH2, MSH6, EXO1, and MLH3 alter hypermutation spectra and impair switch recombination [70–79]. The mechanism that generates DNA breaks in *Msh2*-dependent diversification is not understood. In contrast to UNG-directed mutagenesis, which focuses on C/G pairs, especially at WRCY hotspots, MutSα-directed mutagenesis focuses on A/T pairs, probably reflecting excision that removes the U/G mismatch and neighboring bases. In addition to U/G recognition, MutSα has another function in class switch recombination (Figure 5). G4 DNA structures, formed within transcribing switch regions, are high-affinity binding substrates for MutSα; and MutSα bound to G4 DNA can mediate synapsis of transcriptionally activated switch regions [68].

The major polymerase implicated in mutagenic repair in MutSα-directed hypermutation is the Y-family polymerase, polη, the product of the *XPV* gene, deficient in xeroderma pigmentosum variant disease. Human *XPV* patients and polη-deficient mice show reduced hypermutation at A/T bases [80–83] which argues that this polymerase responds to MutSα-directed processing during hypermutation. The ability of MutSα to stimulate the activity of polη *in vitro* further supports a role for this polymerase in synthesis during hypermutation [69]. Polη may have additional roles in Ig gene diversification. It promotes gene conversion in chicken cells [84] and faithfully extends invading strands of recombinational repair intermediates *in vitro* [85].

How is high-fidelity repair avoided?

Uracil in DNA is a common form of damage, and efficient pathways are dedicated to its faithful repair [86]. Normally, uracil in DNA is excised by UNG initiating high-fidelity repair by the base excision repair pathway [86]. However, AID-initiated deamination of C to U at diversifying Ig genes promotes mutagenesis, not faithful repair. AID is the only B cell-specific factor required [87, 88], and all downstream

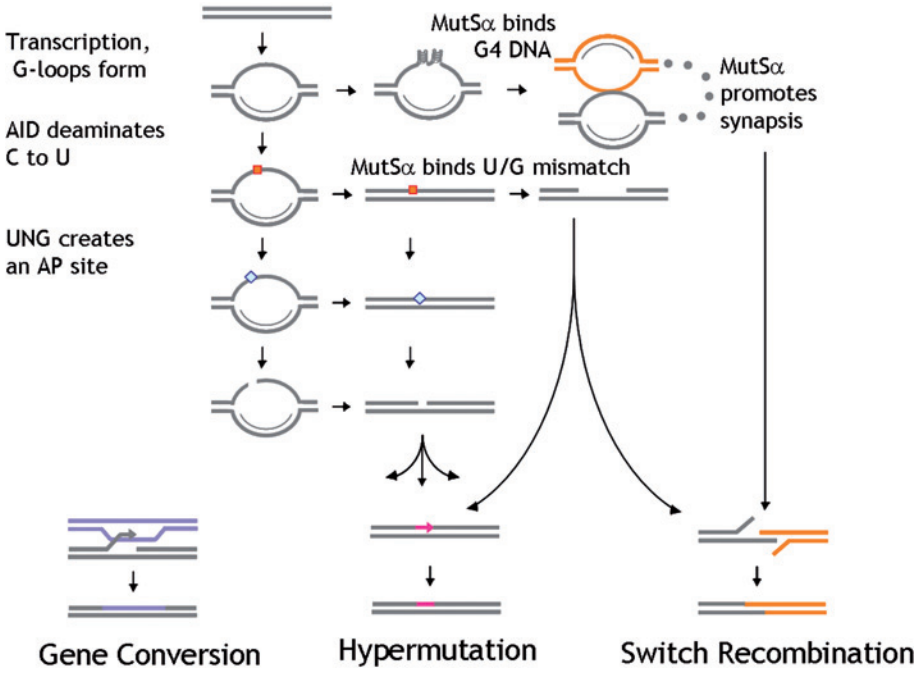

Figure 5. Mechanism of Ig gene diversification. Transcription of Ig genes produces a substrate for AID, and also causes G4 DNA to form within G-rich S regions. Deamination by AID converts C to U (orange box). U can be attacked by UNG to create an abasic site (blue), which is cleaved by the MRE11 AP lyase, to produce single-strand breaks which can be sites of templated (gene conversion), nontemplated (hypermutation) repair or class switch recombination. In a redundant pathway, U/G mismatches are bound by MutSα, which promotes cleavage and mutagenic repair. MutSα also binds to G4 DNA in transcribed S regions to mediate synapsis. See text for additional details.

steps are carried out by ubiquitous factors, which, as described above, assume paradoxically mutagenic roles at the diversifying Ig genes.

It is not currently known whether most of the uracils generated by AID are converted to DNA breaks and mutations, or if they are instead faithfully repaired. This question has recently been addressed both in chicken B cells and in knockout mice. In experiments that monitored C to U transition mutations in *UNG*$^{-/-}$ derivatives of the chicken B cell lymphoma, DT40, approximately 15% of lesions were found to be targets for gene conversion, while the majority of C to U mutations were faithfully repaired [60]. This would lead to the conclusion that faithful repair is predominant, but there is a caveat in the experimental design. AID expression levels were relatively high in the specific derivative of DT40 cells examined, possibly exceeding the capacity of the cells for gene conversion. Two approaches have been taken to examine the extent and localization of AID-initiated damage in murine B cells. Comparison of sequences of Ig V regions from *Ung*$^{-/-}$ and wild-type mice demonstrated indistinguishable levels and localization of C to U transition mutations in the two backgrounds, leading to the conclusion that AID-initiated damage is not faithfully repaired to any significant degree [89]. Examination of C to U transition mutations accumulating at S regions in *Ung*$^{-/-}$*Msh2*$^{-/-}$ mice could not correlate levels of mutation directly with numbers of recombination events, but did provide evidence for processivity of deamination which is not evident in products of mutagenesis, suggesting that mutagenic processing of uracils may interfere with further activity of AID [36].

What makes a gene a target for AID-initiated mutagenesis?

Cis-elements

The possibility that *cis* elements might be important to targeting hypermutation inspired extensive deletional analysis of Ig transgenes with the goal of identifying the key element, with limited success [90, 91]. Notably, E proteins were implicated as potentially important in promoting mutagenesis when a transgene which fortuitously carried multiple matches to the consensus E box recognition site was found to undergo increased levels of hypermutation [92]. E proteins are basic helix-loop-helix proteins, and a subset including E12, E47, E2-2, and HEB are critical to B cell development and activation [93]. E proteins bind to a hexameric sequence which reappears throughout the genome, though not all sites actually function in *cis* regulation. E proteins clearly promote Ig gene diversification somehow: Ig gene diversification is stimulated by E2A expression, and impaired by blocking E protein activity; and these effects seem not to reflect altered transcription levels of the Ig genes [94–97]. However, it is not yet clear whether the effect is direct or indirect. E proteins regulate AID expression in murine B cells [98], which has made it difficult to interpret effects of manipulating their levels in mouse models.

Chromatin structure

Thus far analysis of chromatin structure at diversifying mammalian Ig genes has been limited [44, 99–102]. Histones appear to be hyperacetylated, consistent with very active transcription. Perturbation of histone deactylation by trichostatin A causes an increase in mutagenesis, not only in the V region but extending into the C region [102]. However, this compound affects the entire cell, complicating interpretations of its effects. Conflicting results about localization of specific modified histones at V and C regions have been obtained in different model systems [99, 100]. The question of whether conserved changes in chromatin structure promote or target diversification could be addressed by systematic comparison of chromatin modifications at mammalian and chicken Ig genes.

AID attacks oncogenes to promote translocation

Translocation of c-*MYC* to the Ig heavy-chain locus characterizes B cell lymphomas [103]. There was long-standing debate over whether the prevalence of such translocations reflected the proliferative advantage conferred by deregulation of c-*MYC* resulting from juxtaposition with the potent heavy-chain transcriptional enhancer, or whether translocation depends upon factors that promote Ig gene diversification. c-*MYC* is one of several oncogenes which are targets for aberrant hypermutation in B cell lymphomas; others include *BCL6*, *RhoH*, *PIM1* and *PAX5* [104–106]. The c-*MYC* gene contains a G-rich region, which may be a functional analog of G-rich heavy-chain S regions which are the targets for class switch recombination; and AID binds to this region in transcribed c-*MYC* genes [107]. A requirement for AID in c-*MYC* translocation has been demonstrated in a murine model for Burkitt's lymphoma, where translocations have also been shown to depend upon UNG, one of the factors critical to Ig gene diversification [108, 109]. The evidence that a specific subset of protooncogenes is targeted for aberrant hypermutation in B cell lymphomas [105] suggests that specific features of these oncogenes must be recognized by AID or other factors that promote AID activity. Elucidation of these features should provide valuable insights into the mechanism of AID targeting.

Questions for the future

Recent analysis of AID function has greatly advanced our understanding of the mechanisms that promote Ig gene diversification. Nonetheless, major questions remain unanswered. We do not know how AID is targeted to the Ig genes, nor do we understand how the remainder of the genome escapes mutagenesis. We do not understand how repair factors, normally charged with protecting the genome from mutation, create a paradoxically mutagenic outcome at lesions generated by AID. The field awaits detailed structural and biochemical analysis of AID, to identify the factors and mechanisms that dictate Ig gene diversification and at the same time spare other genes from deleterious mutations.

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