Immunoglobulin Somatic Hypermutation

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Key Words

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Abstract

The immunoglobulin (Ig) repertoire achieves functional diversification through several somatic alterations of the Ig locus. One of these processes, somatic hypermutation (SHM), deposits point mutations into the variable region of the Ig gene to generate higher-affinity variants. Activation-induced cytidine deaminase (AID) converts cytidine to uridine to initiate the hypermutation process. Error-prone versions of DNA repair are believed to then process these lesions into a diverse spectrum of point mutations. We review the current understanding of the molecular mechanisms and regulation of SHM, and also discuss emerging ideas which merit further exploration.

Ag: antigen

Ig: immunoglobulin

SHM: somatic hypermutation

CSR: class switch recombination

GC: gene conversion

INTRODUCTION: IMMUNOGLOBULIN DIVERSIFICATION

There are times when the requirements of biological function exceed the information content of the genome. B-lymphocytes of the adaptive immune system face just such a dilemma: how to extract a virtually infinite repertoire of antigen (Ag)-recognition from a finite supply of genomic information. A "one gene to one Ag" library is unfeasible, so instead, repertoire diversity is achieved through somatic alterations of the immunoglobulin (Ig) locus, which encodes the cell surface receptor responsible for antigen recognition. Ig molecules contain two light chains and two heavy chains arranged in a roughly Y-shaped configuration. The N-terminal prongs (Variable, or V region) specify the Ag-recognizing capacity, whereas the C-terminal stem (Constant, or C region) specifies the effector functions of the molecule. Somatic diversification events occur at both portions of the Ig.

Primary diversification occurs in early B cell development during assembly of the V region, a process called V(D)J recombination. This involves the joining of three segments—Variable (V), Diversity (D), and Joining (J)—randomly selected from a germline pool of multiple gene segments (**Figure 1**). A mature B lymphocyte which has undergone V(D)J recombination is then distinct from all others at three levels: (*i*) the choice of V, D, and J segments, (*ii*) the combination of rearranged heavy and light chains, and (*iii*) junctional insertions and deletions which occur during rearrangement.

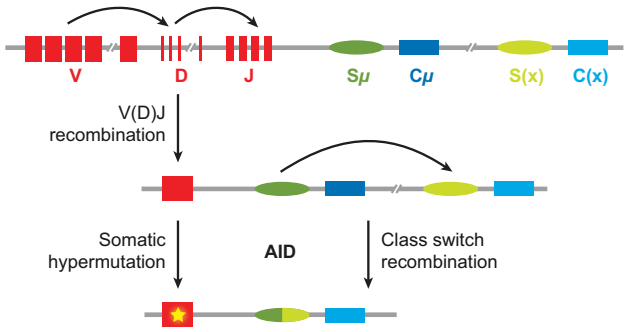

Figure 1

Ig diversification events. Schematic of the mouse IgH locus (not drawn to scale) and somatic alterations of the locus. Developing B cells assemble a functional V region from a pool of germline V, D, and J gene segments [V(D)J recombination]. Further diversification events later occur in an AID-dependent manner. SHM at the rearranged V region produces variants with increased antigen affinity. Secondary Ig isotypes are produced through CSR, which replaces the initial Cμ with any downstream Cx region (where "x" is γ3, γ1, γ2b, γ2a, ε, or α) via nonhomologous recombination between S regions preceding the C exons. SHM and CSR are depicted here as simultaneous events, but in vivo they occur independently of one another.

After maturation in the bone marrow, B cells expressing functional Ig migrate to the periphery, where Ag-dependent secondary diversification may take place. Somatic hypermutation (SHM) introduces point mutations into the V region antigen-binding pocket, creating Ig variants with enhanced affinity for a particular Ag. These mutations arise at a rate of 10^{-3}/basepair/generation (52), several orders of magnitude above the rate of spontaneous mutation. Class switch recombination (CSR), in contrast, exchanges the initial Cμ for one of the downstream C regions through deletional recombination, allowing for switch between different Ig isotypes, each with distinct effector function (**Figure 1**). Certain species such as chickens and rabbits employ gene conversion (GC) as an alternate means of Ig diversification, where the rearranged V gene exchanges short pieces with upstream V pseudogene segments. Though mechanistically disparate, SHM, CSR, and GC share a common initiation event—deamination of cytidine to uridine. In this review we focus on SHM—the initiating lesion, the quality of lesion resolution that leads to mutation, and the protein factors and locus elements that regulate the process.

CHARACTERISTICS OF SHM

Transcription Dependence of SHM

Point mutations introduced into the V region reside within a limited physical range. The domain of mutation begins ~150 bp

downstream of the IgV promoter, and extends for ∼1–2 kb, exhibiting decreasing mutation frequency with increasing distance from the promoter (50). This pattern suggests that the promoter plays a role in the positioning of mutations in the V region; and indeed, SHM has been shown to be intimately linked to transcription. Deletion of the promoter in an IgH transgene results in diminished SHM (18), and furthermore, the rate of mutation directly correlates to the amount of transcription through a locus (2).

Storb and colleagues have formulated a model to describe this concurrence of SHM and transcription, in which a mutator factor associates with an RNA polymerase II (RNAP) transcriptional complex, and deposits mutations as it tracks along the transcribed gene (27, 43). The absence of mutations in the 5′-most ∼150 nts could be explained by two nonmutually exclusive mechanisms: (*i*) the RNAP complex must transition to the elongation phase of transcription before the mutator can dock onto the complex, and/or (*ii*) the transcriptional complex must process a certain distance into the gene to make the mutational substrate available to the mutator (for example, a wake of supercoiled DNA trailing behind an RNAP complex). Then, mutation would begin to trail off as the transcriptional complex travels away from the promoter simply due to natural "breathing" of the mutator-RNAP complex, or perhaps due to more active processes of dissociation (for instance, during RNAP pausing).

Transcription is clearly necessary, but not sufficient, for SHM to occur. With a few exceptions (38, 42), non-Ig genes are not hypermutated even when highly transcribed. A more active targeting process must be at work to target the Ig gene for SHM, and furthermore, to deliver mutation machinery specifically to the V region of the Ig locus. The nature of this targeting process remains a mystery.

Molecular Spectrum of SHM

Along with its physical boundaries, SHM also shows specific molecular properties. Mutations at all four bases have been observed, with transitions predominant over transversions (19). The local environment can specify the mutability of certain bases. C:G basepairs appear to mutate at equal frequencies regardless of strand placement (template vs nontemplate); however, strand polarity is observed for A:T basepairs, where mutation preferentially occurs on a template strand A (34). Primary sequence may affect the placement of mutations, as mutated Cs are often seen in conjunction with a hotspot motif, WRCY (where W = A/T, R = A/G, and Y = C/T), or its complement RGYW (53). Hotspot motifs are not absolute determinants of mutability, however, since not all mutated Cs are associated with hotspots. Additionally, the primary sequence of the V region is not definitively required for SHM, as it can be replaced by a heterologous gene with no apparent defect in mutation rate (68).

These observations give rise to the following questions: What accounts for the qualitative differences in mutation at C:G vs A:T? What unique property does the IgV locus possess, other than primary sequence, which could render it more amenable to SHM than all other genes?

Transition: a purine to purine (A or G), or pyrimidine to pyrimidine (C or T) base change

Supercoiled DNA: DNA compacted by changes in helical twisting

Transversion: a purine to pyrimidine base change, or vice versa

AID: activation-induced cytidine deaminase

ACTIVATION-INDUCED CYTIDINE DEAMINASE

For four decades after Lederberg first proposed that "genic diversity of the precursors of antibody-forming cells arises from a high rate of spontaneous mutation" (26), the identity of the mutator remained unknown. It wasn't until 1999 that Honjo and colleagues identified activation-induced cytidine deaminase (AID) as the key factor that triggers not only SHM but also CSR (35, 36). Gene conversion was later shown to be dependent on AID as well (1), implicating AID as a fundamental mediator of Ig diversification processes.

APOBEC: apolipoprotein-B editing complex

Protein kinase A (PKA): a cAMP-dependent serine/threonine protein kinase with a consensus phosphorylation site RRx(S/T)

ssDNA: single-stranded DNA

Features of AID

Based on sequence homology, AID was classified into the APOBEC family of polynucleotide cytidine deaminases, which perform hydrolytic deamination of cytidine (C) to uridine (U). Much like its APOBEC relatives, AID contains a canonical cytidine deaminase motif, with key histidine and cysteine residues used for zinc coordination and catalysis. The positively charged N terminus carries a putative bipartite nuclear localization signal, though its nuclear localization capacity has not been definitively shown (21, 32). The C terminus, in contrast, harbors a leucine-rich nuclear export signal, which accounts for the predominantly cytoplasmic distribution of AID protein (9, 21, 32). Mutational studies have provided evidence of further functional polarity between the two termini of AID. N-terminal AID mutants are feeble hypermutators, but retain CSR-competence; conversely, C-terminal AID mutants are SHM-competent, but show impaired CSR (5, 58). These observations imply the existence of SHM- or CSR-specific cofactors that preferentially associate with AID at the N terminus or C terminus, respectively, and confer upon it distinct targeting or functional capacities.

In addition to regulation of AID via these intrinsic features, posttranslational modification also modulates AID behavior. Protein kinase A (PKA)-dependent phosphorylation of serine-38 (S38) occurs on a small subset of AID molecules in activated murine B cells (6, 33, 41). Mutation of this phosphorylation site results in functional impairment of both SHM and CSR (6, 33, 41). However, non-S38-phosphorylated AID demonstrates enzymatic competence (7), so the modification appears to be regulatory, not activating. Indeed, S38 phosphorylation is essential for the association between AID and one of its few known interacting partners, replication protein A (RPA), a single-stranded DNA (ssDNA) binding protein (6, 10). This observation has driven the hypothesis of RPA-mediated delivery of AID to a preferred ssDNA target. An additional phosphorylation site at tyrosine-184 has been experimentally observed, and a putative site at threonine-27 lies in a PKA consensus motif, but the functional significance of these residues has yet to be confirmed.

APOBEC family members have been shown to form functional homodimers, and AID appears to be no exception. Mutational experiments have pinpointed the region between threonine-27 to histidine-56 as an essential dimerization motif (62). Comparable conclusions have also been drawn from comparative modeling of AID based on a recently published APOBEC2 crystal structure, though this latter study also proposes an AID tetramerization interface (47). To date, the crystal structure of AID itself has not been determined, leaving comparative modeling as an informative, but in many ways, flimsy substitute for structural information about AID. Technical difficulties in obtaining workable quantities of soluble and biochemically "well-behaved" recombinant AID have greatly hampered progress on this issue. Nevertheless, solving the AID structure remains a necessary undertaking, as the physical form of the enzyme should potentially uncover secrets of molecular function.

Biochemical Activity of AID

Noting the homology between AID and the well-characterized RNA-deaminase APOBEC1, the original discoverers of AID proposed that it edited and activated the mRNA of an SHM- or CSR-catalyzing factor. Though some intriguing arguments have recently been made in favor of this RNA-editing hypothesis for CSR, the bulk of experimental evidence endorses a DNA-deaminating role for AID in secondary Ig diversification.

Ectopic expression of AID in *Escherichia coli* allows for widespread genomic mutation, and since bacteria are rather unlikely to host the same complement of mRNA substrates as

vertebrate B cells, this implies direct modification of DNA (44). The same authors showed that AID-induced mutation in *E. coli* can be enhanced by deficiency of uracil DNA glycosylase (UNG), a base-excision repair enzyme that removes uracil from DNA. Accordantly, in vitro demonstrations of AID activity revealed a substrate preference for ssDNA, and not dsDNA or RNA (8, 11, 16, 59).

The intimate correspondence between SHM and transcription suggests a possible mechanism of ssDNA substrate generation, since small transient loops of ssDNA arise within the context of transcription bubbles. Alt and colleagues have hypothesized that RPA binding stabilizes transcription-dependent ssDNA segments, and also delivers AID to its appropriate substrate (10). Transcription-dependent alteration of DNA topology may also contribute to substrate accessibility, as AID preferentially deaminates negatively supercoiled DNA (which is found in the wake of an elongating polymerase) over relaxed DNA (56). Bisulfite sequencing of IgV has revealed that transcription-dependent ssDNA patches certainly exist in vivo, but are maintained only in a chromatinized (i.e., protein-DNA) context (54). Observation of single-stranded patches on both template and nontemplate strands led the authors of this study to speculate on a role for bidirectional transcription through IgV during SHM, particularly with regards to stabilization of ssDNA after collision of two polymerase complexes traveling in opposite directions (54).

In spite of the term "somatic hypermutation," AID is not an intrinsically vigorous mutator. We have already noted the robust nuclear exclusion of AID, so by subcellular localization alone, most cellular AID is sequestered from its substrate, possibly in an inactive state. In vitro, an AID molecule binds with nanomolar affinity to a synthetic substrate carrying a single-stranded bubble, with the enzymatic consequence that it can perform only one deamination every four minutes (24). The resulting long-lived AID-substrate complex could reflect a second role for AID—that of a recruiting platform for proteins that function downstream of the initiating deamination reaction. This scenario is consistent with numerous observations (and inferences) of AID processivity (24, 45, 56). However, a recent study has demonstrated that under conditions of substrate excess, AID functions in a distinctly distributive mode (distributive enzyme), in striking comparison to APOBEC3G, a known processive deaminase (processive enzyme) (12). Which of these models is the more accurate reflection of in vivo AID function remains unclear.

Distributive enzyme: one that catalyzes a single event per substrate

Processive enzyme: one that catalyzes multiple successive events on the same substrate

In addition to the innate "lethargy" of AID, there is some suggestion of active negative regulation in B lymphocytes. Ectopic expression of AID in non-B cells induces SHM, but in a more promiscuous manner than observed in normally hypermutating V regions (28, 69). This difference could be explained by negative regulatory factors present only in the B lymphocyte (one of the few physiological environments of AID), which limit AID activity to the Ig locus. Constitutive overexpression in mice causes development of T-lymphomas, but not B-lymphomas, suggesting again that B-lymphocyte-specific cofactors check the activity of AID, and in this instance play a protective role against B-lymphomagenesis (39). Moreover, comparison of endogenous AID to constitutively overexpressed AID in B cells revealed that the latter performed far less efficiently in SHM (37).

Revelations of AID function have indisputably advanced our understanding of Ig diversification processes. Nevertheless, the means of regulating AID—whether by nucleo-cytoplasmic shuttling, posttranslational modification, or interaction with unidentified partners—remains one of the most actively investigated areas of the SHM field.

RESOLVING THE U:G LESION

AID-mediated conversion of C to U is in itself mutagenic, as simple replication over the site (with recognition of U as T) would yield

transition mutations. Direct consequences of AID deamination, however, cannot exclusively account for the diverse spectrum of mutations observed in vivo. The SHM process instead uses the initiating U:G lesion to commandeer natural DNA repair pathways for mutagenic purposes. The mainstream model for SHM holds that base excision repair (BER) broadens the range of mutations at C:G basepairs, and mismatch repair (MMR) extends the mutation process to A:T basepairs (**Figure 2**).

Base excision repair (BER): a repair pathway that uses DNA glycosylases to remove damaged or inappropriate single bases

Mismatch repair (MMR): a repair pathway that senses and repairs basepair mismatches, usually involving subsequent patch synthesis

BER-Mediated Mutation at C:G

Though not normally present in DNA, U nucleotides occasionally appear as unwelcome guests after AID-independent cytidine deamination or misincorporation of dUMP during replication (23). Base excision repair (BER) faithfully mends these lesions, using one of several uracil DNA glycosylases to remove the offending U. Hypermutating B cells, on the other hand, borrow the uracil DNA glycosylase UNG to facilitate mutation at C:G basepairs. The prevailing hypothesis is that UNG-mediated cleavage of the U base from the DNA backbone results in a noninstructive abasic site, replication of which allows for transversions in addition to transitions. In agreement with this model, UNG deficiency shifts the mutation spectrum in favor of transitions at C:G (15, 20, 51). Additionally, error-prone polymerases may play a role in generating C:G mutations. Genetic evidence suggests that the translesion polymerase Rev1

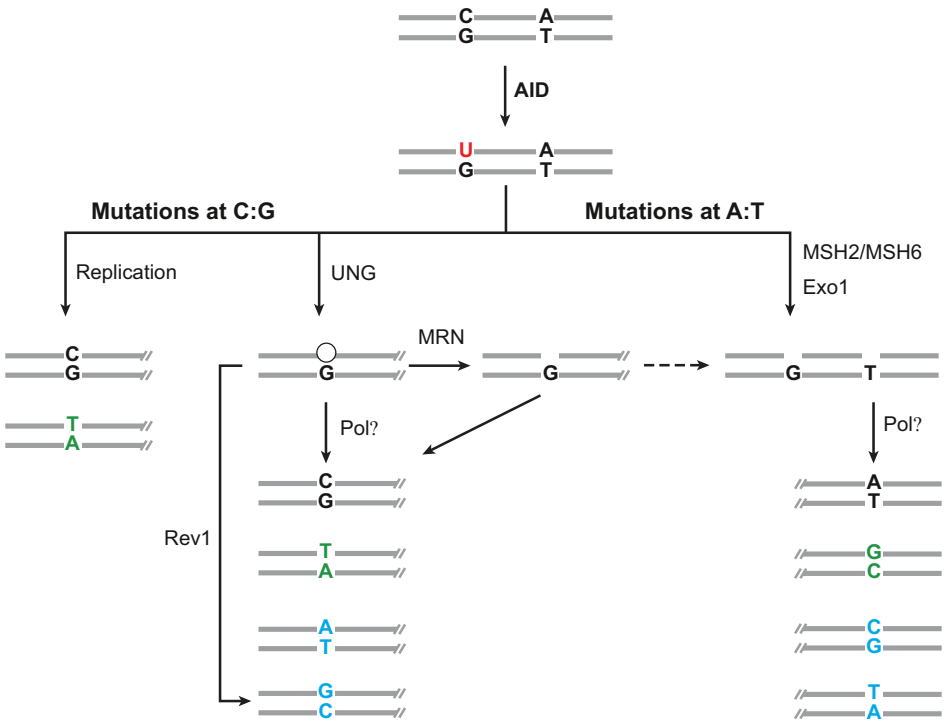

Figure 2

DNA deamination model of SHM. The AID-induced uracil (*red*) in DNA is thought to undergo differential processing to generate mutations at C:G and A:T basepairs. Candidate enzymes regulating the steps after AID-mediated deamination are noted. Transition mutations are indicated in green, and transversion mutations in blue. Cooperation between the BER- and MMR-mediated pathways may also occur (*dashed arrow*).

traverses UNG-induced abasic sites to promote transversions at C:G pairs (22), and that the low-fidelity polymerase θ (polθ) can introduce both transitions and transversions at abasic sites (31).

These observations provide a rational framework for explaining the mechanisms of mutagenesis at C:G pairs, but do not explain why these processes are rendered preferentially mutagenic instead of corrective.

DNA Cleavage and Mutagenesis

A second mutagenic process has been proposed to function in collaboration with the UNG-mediated pathway of mutagenesis. The Mre11/Rad50/Nbs1 (MRN) complex normally functions in DNA break sensing and repair, but its components have also been implicated in SHM. Overexpression of the Nbs1 subunit in a hypermutating B cell line increases the amount of mutation (66), suggesting that the MRN complex can facilitate mutagenic processing of AID-generated lesions. A complementary study showed that Mre11 is enriched at IgV in hypermutating B cells, and furthermore, Mre11/Rad50 cleaves abasic sites to form ssDNA breaks, leaving ends that cannot be extended by the usual high-fidelity DNA polymerases (25). This could then necessitate the use of alternate low-fidelity (i.e., mutagenic) polymerases to further process the damaged site.

MMR-Mediated Mutation at A:T

AID induces two abnormal features into the V region, the first being a foreign base in DNA (discussed above), and the second being a U:G mismatch. Genetic evidence suggests that the latter triggers an MMR-based pathway of mutagenesis, though many details are not understood with great precision. The MMR heterodimer MSH2/MSH6 has been shown to bind U:G mismatches (64), and presumably enables gap formation and error-prone synthesis to generate mutations outside of the initial mismatch. Mice deficient in MSH2 or MSH6 exhibit substantial reduction of A:T mutations (29, 46, 49, 63), supporting the idea of subverted MMR as a major source of mutations at A:T pairs.

An attractive candidate for subsequent gap formation is Exonuclease1 (Exo1), a known partner of MSH2. It has been observed in physical association with the V region, and Exo1-deficient mice also possess the reduced A:T mutation phenotype observed in the MSH2/MSH6 knockouts (4). The single-stranded gap created by ExoI is believed to undergo low-fidelity regeneration by error-prone polymerases, thus extending mutations to A:T basepairs. Compelling evidence implicates the known low-fidelity DNA polymerase η (polη) as the mutagenic culprit. Again, mouse knockout models show that polη deficiency results in diminished mutation of A:T pairs (13, 14, 30), and with allusion to upstream events, MSH2/MSH6 has been shown to stimulate polη catalysis in vitro (64). Other low-fidelity polymerases with mismatch extension capability, such as polθ (71) and polζ (70) are proposed to function in this phase of SHM as well. It is unknown if this repair pathway is capable of introducing the A:T mutation strand bias observed in vivo.

The means by which IgV is temporally and spatially targeted for error-prone repair is largely undescribed. A recent study, though, makes the tantalizing proposal that Rad6/Rad18-mediated recognition of AID-induced DNA lesions triggers the monoubiquitination of proliferating cell nuclear antigen (PCNA), thus signaling for the recruitment of error-prone polymerases (3). Such a DNA-lesion-dependent molecular switch between error-free and error-prone synthesis fits quite nicely into the prevailing model of SHM, and warrants further examination.

Alternate Modes of SHM?

The popular model of SHM as a collaboration between subverted BER and MMR is certainly supported by experimental evidence. Indeed, simultaneous inactivation of

ADAR: Adenosine deaminase acting on RNA, which deaminates adenosine to inosine in dsRNA

UNG/MSH2 or UNG/MSH6 erases the mutational signature of SHM (48, 57). However, some intriguing observations suggest novel amendments or additions to the current model, particularly with regard to mutation at A:T basepairs.

The hypermutating Burkitt lymphoma cell line Ramos performs very little A:T mutation, yet harbors fully functional MMR machinery (MSH2, MSH6, and polη) (65). This could be sensibly explained by the absence in Ramos of an unidentified factor necessary for the canonical MMR-mutagenesis pathway. Conversely, is it possible that MMR per se is not the requirement for mutation at A:T? In either case, additional unknown factors could be at work in this A:T phase of hypermutation.

Completely different mechanisms for A:T mutagenesis have also been recently set forth. Franklin & Blanden comment that the strand-unbiased mutation at C:G vs the strand-biased mutation at A:T is not easily explained by the current model (17). They propose a novel mechanism of RNA-dependent DNA synthesis by polη to introduce mutations specifically at A:T pairs. Along similar lines, a computational study has proposed ADAR-mediated A to I (inosine) editing of IgV mRNA, with subsequent reverse transcription by polη to generate A:T mutations (60). Though clearly more complicated than the MMR-based model, such a mechanism could potentially account for the observed A:T strand bias. These obvious departures from current thinking are not necessarily incompatible with the standard model and could perhaps play alternate or backup roles in SHM; these possibilities will of course require rigorous experimental testing.

REGULATION AND TARGETING OF SHM

SHM run amok can introduce mutations into proto-oncogenes, and such mistargeted mutations have been found in association with a number of B cell lymphomas (42, 55). To avoid these types of deleterious alterations, the mutational machinery must be precisely delivered to the Ig locus. The exact qualities of the IgV region that make it preferentially attractive for hypermutation are not known.

Given that the V region sequence itself is not requisite for SHM, targeting functions for associated *cis*-elements have been proposed. Replacement of the IgV promoter with a heterologous promoter exhibiting equivalent or higher transcriptional activity could not reproduce wild-type levels of SHM (67). This could imply an additional transcription-independent role for the IgV promoter in recruiting SHM to the locus. Similarly, Ig enhancers have been suggested to function as loading docks for targeting proteins and/or AID, in addition to regulating transcription. Enhancer deletions in Ig transgenes have hinted at nontranscriptional roles, but synonymous deletions at endogenous Ig loci have not consistently recapitulated the transgene phenotypes (reviewed in 38). An intrachromosomal delivery system for SHM to IgV based on *cis*-element-mediated recruitment of mutational factors is an appealing hypothesis, but will require more coherent data for validation.

Instead of a defined genetic locus made of a promoter, enhancers, exons, and introns—perhaps the SHM apparatus perceives IgV as a collection of mutationally palatable molecular motifs. Are there primary sequence elements outside of the IgV exon that attract *trans*-factors? What types of secondary structures are prevalent in IgV? Are such motifs mimicked in the oncogenes that are mistarged by SHM? Does chromatin remodeling (histone replacement, for example) temporally and spatially differentiate IgV from other locations in the genome? Further exploration of these questions could potentially reveal the physical signature of IgV that attracts SHM.

An alternate paradigm for targeting is suggested by observations of SHM outside of the Ig locus. We have already noted the occasional mistargeting of non-Ig genes associated with lymphomagenesis (42, 55). Two groups have also reported hypermutability of a proviral

reporter gene regardless of genomic integration site (40, 61). In both of these situations, mutations are far less abundant in comparison to normal SHM at IgV. One could imagine that the entire genome has equivalent potential for mutability by AID, but non-Ig sites are shielded from SHM by the presence of normal high-fidelity DNA repair. Perhaps focusing of SHM to IgV is accomplished not by directed targeting of AID, but by specific delivery of error-prone repair.

PERSPECTIVE

Evolutionary conservation of SHM among diverse vertebrate species, along with the known immunological defects associated with AID-deficiency, reflect the might of SHM, a process that has harnessed the power of mutation to confer immunological protection. Less than a decade after the landmark discovery of AID, a model of SHM as the consequence of subverted DNA repair has been largely embraced by the field. However, our knowledge of the events preceding AID-mediated deamination (targeting, in particular), and the means by which a simple uracil in DNA is permuted into the diverse spectrum of in vivo SHM, still remain incomplete. Refinement of the mainstream model will be necessary to account for unexplained phenomena in SHM. Furthermore, a better understanding of SHM itself will provide insight into oncogenesis, genomic integrity, DNA damage repair, and the related diversification processes of CSR and GC.

SUMMARY POINTS

1. AID-mediated deamination of cytidine to uridine initiates SHM.
2. Mutations at C:G basepairs are believed to arise from error-prone BER, and possibly from MRN-dependent single-strand DNA breaks.
3. Mutations at A:T basepairs are believed to arise from error-prone MMR, though the current model does not account for the strand-biased nature of mutations.
4. Targeting of SHM to IgV is not well understood. Candidate mechanisms include facilitated delivery of AID, recruitment by *cis*-elements or other physical features of IgV, or recruitment of error-prone repair.

FUTURE ISSUES

1. What is the crystal structure of AID, and will it reveal details of catalysis or regulation?
2. How is AID activity regulated—through protein partners, posttranslational modifications, nuclear exclusion, association with specific forms of RNAP?
3. Why does repair proceed in an error-prone fashion specifically at the IgV locus?
4. Are subverted BER and MMR sufficient to explain the molecular spectrum of SHM? Could alternate mechanisms such as RNA-editing play a role?
5. Do *cis*-elements or other intrinsic properties of the IgV locus (sequence motifs, higher-order DNA structures, chromatin structure) predispose it to hypermutation?
6. What are the means of inter- and intralocus targeting of SHM?

DISCLOSURE

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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