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Current Topics

Reward versus Risk: DNA Cytidine Deaminases Triggering Immunity and Disease†

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ABSTRACT: The enzymatic deamination of cytosine to uracil, using the free base C, its nucleosides, and nucleotides as substrates, is an essential feature of nucleotide metabolism. However, the deamination of C and, especially, 5 methyl C on DNA is typically detrimental, causing mutations leading to serious human disease. Recently, a family of enzymes has been discovered that catalyzes the conversion of C to U on DNA and RNA, generating favorable mutations that are essential for human survival. Members of the Apobec family of nucleic acid-dependent cytidine deaminases include activation-induced cytidine deaminase (AID) and Apobec3G. AID is required for B cells to undergo somatic hypermutation (SHM) and class switch recombination (CSR), two processes that are needed to produce high-affinity antibodies of all isotypes. Apobec3G is responsible for protection against HIV infection. Recent advances in the biochemical and structural analyses of nucleic acid cytidine deaminases will be discussed in relation to their programmed roles in ensuring antibody diversification and in imposing innate resistance against retroviral infection. The serious negative consequences of expressing Apobec deaminases in the wrong place at the wrong time to catalyze aberrant deamination in "at risk" sequences will be discussed in terms of causing genomic instability and disease.

Dating from the early 1960s, studies have shown that the enzymatic deamination of cytosine to form uracil plays a central role in salvaging and recycling nucleotide precursors used in the synthesis of RNA and DNA. Cytidine deaminases (CDAs)[1] that catalyze C → U conversion using the base C, its nucleosides, and nucleotides were identified (*1*, *2*). C deaminations on DNA, nearly all of which have been presumed to arise spontaneously, are a major source of C → T transition mutations (*3*, *4*). Most organisms take great pains to eliminate U on DNA by engaging uracil glycosylase (UDG), abasic endonuclease, and other enzymes that act together as part of a tightly choreographed base excision repair (BER) pathway (*5*−*7*). U·G mispairs are likely to be recognized by the mismatch repair (MMR) complex MSH2−MSH6 (*8*, *9*), and there are even specialized MMR glycosylases that eliminate T·G mispairs caused by deamination of 5MeC (*10*, *11*).

On the basis of the efficient elimination of U, one might not have anticipated the need for a biochemical pathway designed for the express purpose of creating U in DNA by enzymatic deamination of C. Yet an example of just such a pathway has recently been reported, one that is essential for immunoglobulin (Ig) affinity maturation in vertebrate B cells. This pathway deliberately causes DNA-dependent conversions of C → U, generating advantageous mutations in the Ig gene that increase the affinity of the antibody for the

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[1] Abbreviations: UDG, uracil DNA glycosylase; AID, activation-induced cytidine deaminase; SHM, somatic hypermutation; CSR, class switch recombination; Ab, antibody; Ag, antigen; BER, base excision repair; MMR, postreplicative mismatch repair; CDA, cytidine deaminase; Vif, virus infectivity factor.

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Table 1: Mammalian DNA Deaminases

DNA deaminase	function	sequence specificity (5′ → 3′) hot spots	cold spots	refs
hAID	adaptive immunity	WRC	SYC	*47*
	SHM and CSR	WRC		*133*
mAID		WRC		*136*
hApobec3G	innate immunity	YCC	WRC	*137*
	antiretroviral	YCC		*106, 107*
	G → A hypermutation	CCCA		*104, 138*
		CCCG		*133*
hApobec3F		TTC		*106*
		(C/A/T)TC		*107*
		TTCW		*104*
hApobec3B		STC		*107*
		YC		*111*
hApobec3C		no preference		*111*
mApobec3		TYC		*107, 138*
Apobec1	apoB mRNA editing	TC		*133*

antigen. Activation-induced cytidine deaminase (AID) is the key to this pathway.

AID is a member of a family of Apobec cytidine deaminases that act on DNA or RNA to convert cytosine to uracil (Table 1). These enzymes have evolved from an ancient class of zinc-dependent multisubunit cytidine deaminases (CDA) that are found in all organisms. The Apobec family of proteins is present in only eukaryotes. Apobec1 ("apoB" editing catalytic subunit 1), the first member of the nucleic acid-dependent CDA family to be discovered, is an RNA deaminase. Apobec1 converts C → U in apolipoprotein B mRNA at a specific nucleotide (nucleotide 6666), converting a CAA to UAA termination codon to produce two forms of ApoB from the same gene (truncated apoB48 and full-length apoB100). Although the physiological function of Apobec1 is to edit mRNA, it can also deaminate C in DNA when overexpressed in *Escherichia coli* (*12*). Other members of the Apobec family, Apobec3G and -3F, are available for protection against HIV infection by triggering the destruction of HIV-1 reverse-transcribed DNA. By modifying DNA, AID, Apobec3G, and Apobec3F play an essential role in survival.

The positive aspects of C → U conversion resulting from the regulated action of Apobec enzymes have just begun to be addressed on cellular and biochemical levels. The negative aspects of C → U conversions raise an interesting question concerning the extent to which the unregulated deamination of C occurs spontaneously compared to the inappropriate action of DNA cytidine deaminases. Recent comprehensive reviews portray the biological roles of AID during antibody diversification (*13*−*16*) and the antiretroviral role of Apobec3G (*17*−*21*). This review emphasizes the biochemistry of AID, the antiviral properties of Apobec3G and -3F, and the attendant rewards and risks of programmed versus nonprogrammed DNA deamination.

BIOCHEMICAL ROLE OF AID IN ANTIBODY DIVERSIFICATION

The production of the high-affinity antibody (Ab) depends on the action of AID (*22*). AID is induced in B cells, during a limited time span, to generate U intermediates required to produce Ig antibodies with high-affinity antibody (Ab)−antigen (Ag) recognition. In humans and mice, the DNA sequences of Ig genes undergo diversification by three processes: V(D)J recombination, somatic hypermutation (SHM), and class switch recombination (CSR) (Figure 1). V(D)J recombination involves the rearrangement of the Ig V, D, and J gene segments in producing a unique antigen binding site. A second stage of diversification is triggered by antigen binding and occurs in germinal center B cells that express AID. During this stage, B cells undergo affinity maturation via SHM and CSR (Figure 1). SHM involves the generation of point mutations in the V regions of the Ig gene to optimize Ab−Ag binding affinities. CSR converts Ig molecules into different isoforms by recombining the rearranged V(D)J region with one of eight different heavy chain C regions to convert an IgM isotype into IgA or IgE or into one of the four IgG isotypes. Simply put, CSR uses nonhomologous recombination to express downstream constant regions (*23*). Each Ab isotype has a distinct effector function designed to trap, neutralize, and clear Ag molecules from each of the body compartments. Together, SHM and CSR finely tune the Ab−Ag recognition process by converting low-affinity to high-affinity antibodies that can carry out all possible effector functions. SHM and CSR both require AID (*24*). AID deficiency in humans causes an inherited immune disorder, Hyper-IgM2 syndrome (*25*). Patients with Hyper-IgM2 often have normal or elevated serum IgM levels but do not have detectable serum levels of IgG, IgA, and IgE (*26*), nor do they have V gene mutations (*25*).

The discovery of AID by Honjo and co-workers was made using a subtractive hybridization screen for genes activated in B cells, where the induction of CSR resulted in a 10-fold increase in the extent of AID expression (*24*). Subsequent gene targeting experiments showed that homozygous mutant mice lacking AID had normal B cell maturation but were deficient in both CSR and SHM. Overexpression of AID in CH12F3-2 and Ramos B cells, and even in fibroblasts, was sufficient to activate CSR and SHM (*22*, *27*−*29*). These studies demonstrated that AID is essential for initiation of SHM and CSR (Figure 1).

Several lines of evidence showed that AID deaminates C on ssDNA. The overexpression of AID in *E. coli* (*30*) and human cell lines (*27*, *28*) caused elevated levels of C → T mutations, suggesting that AID is likely targeting DNA. Cell free assays provided direct biochemical evidence that AID may be acting solely on ssDNA (*31*−*34*). No measurable level of deamination was observed on dsDNA, RNA, or RNA−DNA hybrids (*31*, *32*). AID was also found to deaminate 5MeC → T on ssDNA at a reduced rate compared to that of C (*31*, *35*, *36*).

Relating AID Biochemistry to SHM and CSR in Vivo

An in vitro biochemical approach can facilitate the unraveling of many of the opaque properties of SHM and CSR. The rate of V gene mutations in B cells is 1 million-fold higher ($\sim 10^{-4}$ to 10^{-3} per base pair per generation) than typical somatic cell mutation rates (10^{-9} per base pair per generation). SHM requires active V gene transcription, where mutations tend to occur as a gradient, beginning ~250 nucleotides downstream from the promoter and decreasing over a range of ~2000 nucleotides (*37*−*39*). There are

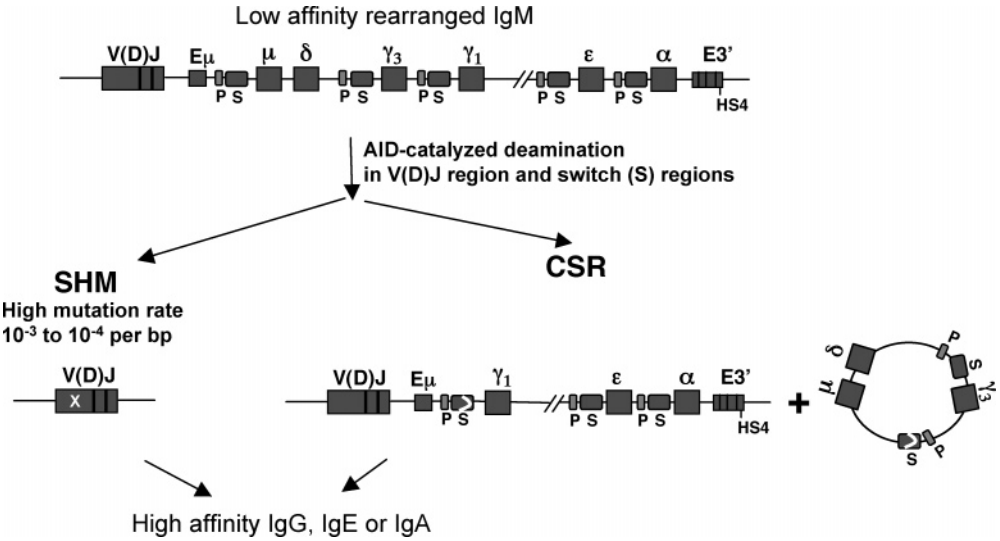

FIGURE 1: Essential role of AID in SHM and CSR. Following V(D)J recombination, which generates an initial low-affinity antibody repertoire by a random rearrangement of the V, D, and J genes, the V(D)J rearranged genes further undergo SHM and CSR for generation of high-affinity antibodies expressing all isotypes. SHM and CSR require deamination by AID in V(D)J and switch (S) regions, respectively. SHM introduces a massive number of missense mutations (X), approximately 1 million-fold more frequently than normal spontaneous mutation, in the antigen-binding V region of the Ig gene. CSR combines the V region of the gene with appropriate downstream constant regions, γ3, γ1 (γ2b and γ2a not shown), α, or ϵ, converting IgM or IgD to the other isotypes, IgG3, IgG1, (IgG2b and IgG2a not shown), IgA, or IgE, respectively. The product of CSR is a deletion of intervening constant regions in the form of looped out circular DNA. P represents promoters adjacent to switch (S) regions. Eμ and E3′ are enhancer regulatory regions. HS4 is an enhancer binding site within the E3′ region that is important for CSR.

roughly equal numbers of mutations on nontranscribed and transcribed strands (*40*). Most of the mutations are single-base substitutions, with transitions slightly favored over transversions. Two prominent mutational hot spots are present in the V gene, WRC (W is A or T; R is purine) and WA (*41*, *42*). These SHM properties are not well understood.

In the case of CSR, transcription is required at switch (S) regions, which are 2−12 kb long and precede each heavy chain C_H gene (Figure 1). Transcription is initiated by an activator/cytokine-specific promoter upstream of each S region and terminated downstream of the corresponding C_H gene, generating noncoding germ-line transcripts. There are strong indications that germ-line transcription of S regions in vivo and in vitro results in the formation of an R-loop structure, in which the germ-line transcripts stably form DNA−RNA hybrids with the transcribed strand leaving the displaced nontranscribed strand exposed as single-stranded DNA (*43*, *44*). This exposed ssDNA is a likely target for AID deamination that in turn could initiate CSR. Subsequent processing of G·U mispairs by BER or by MMR could generate double-stranded break intermediates. Nonhomologous end joining completes CSR (*16*, *45*). In support of this hypothesis, ectopically expressed AID is directly associated with the transcribed S region in cells undergoing CSR (*46*) and AID deamination of transcribed dsDNA has been demonstrated in vitro (*32*, *47*−*49*). In mice that undergo SHM, but not CSR, WRC hot spot mutations are observed within the switch regions where double-stranded breaks would normally occur during active CSR (*50*).

Although the initial steps of SHM and CSR overlap in that both require AID deamination (*22*) and active transcription, the downstream events remain unclear and call for further biochemical explanation. The expression of AID, which occurs during a short time span in B cells, is essential for SHM as is active transcription (*51*, *52*). In fact, biochemical studies with semipurified AID were required to reveal that ssDNA was the substrate for AID and thus perhaps to explain the perplexing need for transcription (*32*, *47*, *48*, *53*).

An in vitro approach that can be used to investigate the specific types and distribution of V gene mutations has emerged recently, facilitated by biochemical studies with AID (*47*, *48*) and with error-prone pol η (*42*). C → U conversion can be assessed at a large number of sites by incubating AID with phage M13 gapped circular DNA substrates containing a *lacZ* ssDNA reporter sequence (*47*, *48*). AID-catalyzed C → U deaminations are detected as C → T mutations in individual phage progeny grown in uracil glycosylase-deficient host cells. The assay can also be used to assess AID deaminations on DNA undergoing active transcription using covalently closed supercoiled M13 DNA, containing the *lacZ* reporter gene located downstream of a T7 promoter (*48*).

AID-Catalyzed Deamination of C Simulates SHM Mutational Specificity. When acting on ssDNA, AID preferentially converts C → U in WRC hot spot motifs while avoiding SYC cold spot motifs (*47*, *48*), suggesting the possibility that C → T mutations in V genes may be targeted primarily, if not solely, by AID. A comparison of the sequence specificity for deamination by AID with V gene mutations in humans can be made by comparing in vitro and in vivo data using a "mutability index", defined as the ratio of the number of times that mutations occur in a specific DNA sequence divided by the expected number of mutations occurring without sequence bias (*54*). The mutability index for AID averaged over all WRC sites, 1.9 ± 0.3 (*47*), agrees with the SHM value of 1.9 ± 0.6 determined in vivo (*54*). Similar agreement is found for SYC cold spots, which have an average mutability index of 0.4 ± 0.3 for AID (*47*) compared to the value of 0.3 ± 0.1 found for humans (*54*). Hot spot targeting, aside from AID, also involves error-prone DNA polymerases (*42*, *55*−*57*). Irrespective of what the cast

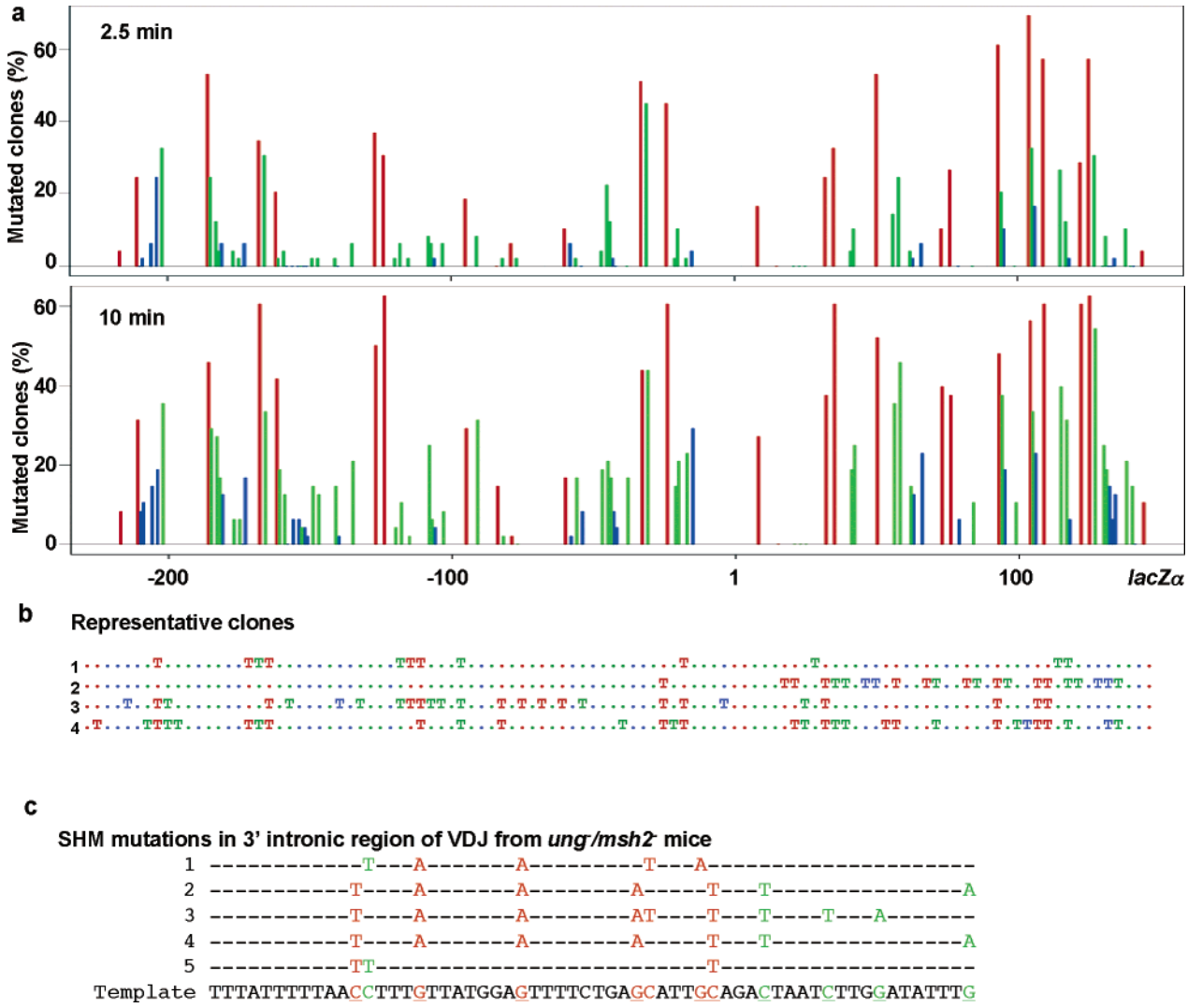

FIGURE 2: AID-catalyzed C deamination specificity on ssDNA. (a) C → U deamination spectra following incubation of ssDNA substrates with wild-type AID for 2.5 and 10 min (*48*). Each colored bar represents a percentage of deaminated substrates with a C → U deamination at the indicated position in the *lacZ*α target sequence. Red bars identify C deaminations occurring in 5′ WRC hot-spot motifs; blue bars represent 5′ SYC cold-spot motifs, and green bars represent neither WRC nor SYC "neutral" motifs (W is A or T, R is a purine, S is C or G, and Y is a pyrimidine). (b) Identification of C → T mutations in individual DNA clones incubated with AID for 10 min. The colored dots show the locations of WRC hot spot sites (red), NNC neutral sites (green), and SYC cold-spot sites (blue) in the *lacZ*α target sequence where mutations have *not* occurred. The colored T's indicate C → T mutations that have occurred at hot spots, neutral spots, and cold spots. (c) SHM mutations in a 3′ intronic region of VDJ in five representative clones from *ung*⁻/*msh2*⁻ mice in vivo (see ref 80). Mutations reflect the deamination action of AID on the nontranscribed strand (G → A) and on the transcribed strand (C → T) at predominantly WRC hot spots (red) and fewer neutral spots (green). The distribution and pattern of mutations are roughly similar to the deamination pattern of AID on naked ssDNA (Figure 2b).

of players ultimately turns out to be, the ability of AID to act in vitro at biologically relevant hot spot motifs underscores its relevance in targeting and initiating V gene mutations.

Spatial and Temporal Patterns of AID-Catalyzed C → U Conversions in Vitro. The *lacZ* reporter assay facilitates an in-depth investigation of AID C deamination spectra, their clonal patterns, and their evolution over time (Figure 2a). Deaminations are favored at WRC hot spots (Figure 2a, red bars) compared to NNC neutral spots (Figure 2a, green bars) and SYC cold spots (Figure 2a, blue bars) at short (2.5 min) and longer (10 min) incubation periods; however, over time, the frequency of mutations at neighboring non-hot spot sites increases, while there is little or no increase in the numbers of mutated clones. AID seems to remain bound to the single-stranded gap region of M13 while deaminating in a processive-like manner. The hot spots appear to serve as nucleation sites for multiple deaminations at nearby non-hot spot C sites (Figure 2b, clones 2−4), yet there are also examples of clustered deaminations separated by regions that suffer little or no deamination (Figure 2b, clone 1). We have observed that AID does not appear to bind preferentially at hot spots, but instead binds with similar affinity to ssDNA, either with or without C (R. Bransteitter, P. Pham, and M. F. Goodman, unpublished results), which suggests a random binding mechanism. Many deamination clusters, although not all, are located within 10 residues of a WRC motif, suggesting that AID may be acting processively over a limited region (*48*). The pattern of randomly clustered deaminations observed for individual DNA molecules in vitro is evocative of the broad clonal mutagenic heterogeneity of V genes (*58*−*60*). An example of clustered SHM mutations in vivo (Figure 2c) roughly resembles the in vitro clonal behavior of AID acting on naked ssDNA (Figure 2b).

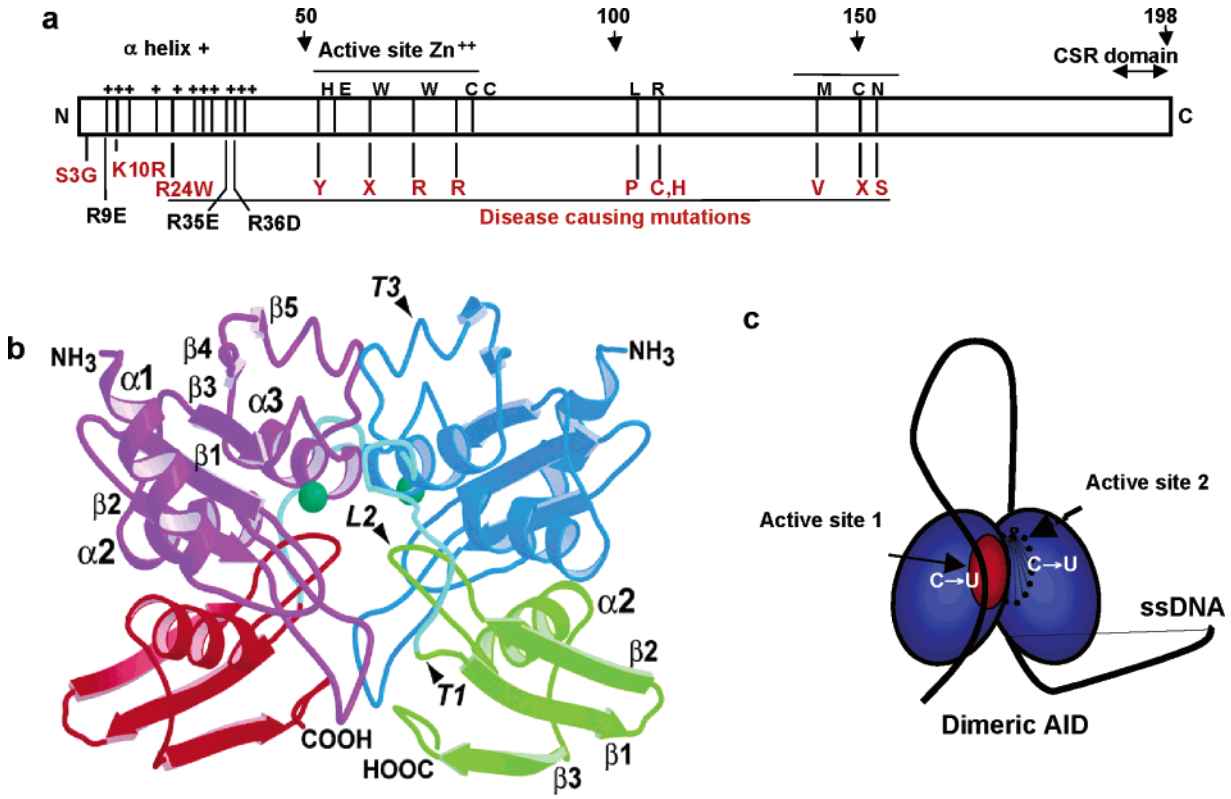

FIGURE 3: Model describing the possible "processive translocation" behavior of AID on ssDNA. (a) Schematic representation of the AID domain structure. (b) A hypothetical AID model based on a structural alignment with yeast CDD1 (*69*). The dimeric "head-to-tail" hypothetical model of AID with N-terminal cytidine deaminase domains (purple and blue) and coordinating Zn^{2+} (dark green sphere). Trans-acting structure elements that form the purple active site are as follows: T1 and L2 (green), central flap (cyan), and T3 (blue). (c) Sketch depicting the possible binding by two active sites of a dimeric AID to the same ssDNA molecule. Localized mutational clusters, as found in representative clones shown in Figure 2b, could occur if each subunit performed processive C deamination over a localized region of ~10 bases on ssDNA.

The properties of AID activity and deamination specificity can be investigated in vitro using site-directed mutagenesis. The N-terminal α-helical region of AID carries a large positive charge (+11) (Figure 3a). Replacement of basic amino acid residues with acidic amino acids, as in the R35E/R36D AID mutant, alters the properties of AID in two important ways (*48*). First, in comparison to wild-type AID, the mutant enzyme shows a smaller increase in the average number of deaminations per clone with time, along with a concomitant increase in the fraction of deaminated clones. Thus, the mutant AID behaves more distributively, by cycling more rapidly between different ssDNA substrates, which is consistent with a reduction in AID processivity (*48*). Second and unexpectedly, mutations in the N-terminal region also altered mutational specificity such that the most prominent C deamination site occurs in a CAC non-hot spot (*48*).

N-Terminal mutants are biologically important. First, several mutations in this region, S3G, K10R, and R24W (Figure 3a), have been found in immunodeficient patients (*61*). The R24W mutation inhibits SHM and CSR in vivo, while S3G and K10R mutations significantly reduce both. Notably, a mutation at amino acid 24 (R24E) renders AID inactive in vitro (*48*). Other SHM-defective mutants have been mapped at several locations in the positively charged N-terminal α-helix (*62*). The biochemical data with N-terminal mutant AID suggest that a reduction in processivity would result in a general reduction in SHM, in accord with the in vivo data (*62*). In contrast, a 10-amino acid C-terminal deletion has essentially no effect on either the biochemical activity or specificity of AID (*48*), but it does result in a deficiency in CSR in vivo (*50*). This deletion is able to induce mutations in the Sμ (switch) region, which suggests that the C-terminus is needed to interact with a CSR specific factor, required for postmutational events (*50*). While progress has been made toward elucidating a biochemical basis for SHM and CSR, a deeper level of understanding awaits a high-resolution structural analysis of Apobec family members. X-ray structures are available for several cytidine deaminases; a recent structure for yeast CDD1 may provide important insights into the mechanistic behavior of AID (Figure 3b,c).

Relating Structural Models of Yeast Cytidine Deaminase CDD1 to AID-Catalyzed Deamination Clusters

Bessman and colleagues (*63*) showed that two enzymatically inactive monomers of *Staphylococcus aureus* dCMP deaminase combine to form an active dimer when bound to dCTP, whereas an inactive dimer is formed with dTTP. This type of duel substrate−inhibitor regulation enables the cell to maintain balanced pyrimidine deoxynucleoside triphosphate pools. Although this regulatory process appears to be specific to *S. aureus* dCMP deaminase, the requirement to form multimers appears to be a general property of C deaminases, including those using DNA or RNA as substrates. To date, AID, Apobec3G, and the other nucleic acid deaminases have been difficult to purify in a sufficiently active form and in quantities sufficient for structural studies.

There are structures available for several deaminases, including *E. coli* dimeric CDA (*64*), *Bacillus subtilis* tetrameric CDA (*65*), *Saccharomyces cerevisiae* CDA (*66*, *67*), dCTP deaminase (*68*), and *Sa. cerevisiae* CDD1 (*69*). The structure of yeast CDD1 is potentially important as a model for nucleic acid C deaminases because it also acts on ssRNA (*70*). A comparison of CDD1 with other CDA structures is instructive. The catalytic domains for all CDAs are structurally similar. Each has five β-sheet strands flanked by three α-helices (Figure 3b). The similar folding of the deaminase domains is characterized by a conserved signature motif, (C/H)xEx$_n$PCxxC. The Cys/His and two other Cys residues coordinate zinc binding at the active site, and the Glu residue is involved in proton shuttling during catalysis.

The structural differences between CDA and CDD1 may be relevant to AID. CDA structures contain a bulky polypeptide flap which folds over the active site entrance, thus effectively excluding substrates larger than a single nucleotide base. In contrast, the flap observed for CDD1 is considerably smaller, to enable larger substrates, e.g., ssRNA, to gain access to the deaminase active site (*69*). Taking the CDD1 structure as a model template, and including the conserved deaminase active site domain, Xie et al. (*69*) proposed a hypothetical model for a dimeric AID in which two monomers are arranged head to head such that their active sites are on opposite sides of the dimer (Figure 3b). In this configuration, each monomer could act on two C moieties on the same, or perhaps different, ssDNA molecules. It appears that formation of the active site may require a flexible trans-acting flap to fold over the active site of the other AID subunit, which could impose active site size constraints as a means of regulating substrate selectivity. A requirement for an intersubunit flap interaction could explain why Apobec homologues, including AID, are active only as multimers. Xie et al. (*69*) propose that, because of steric constraints imposed by the flap-defined active site architecture, single-stranded RNA or DNA can be accommodated, whereas dsDNA would be excluded.

A model for AID based on yeast CDD1, although tenuous, is attractive in the sense that it might shed light on the unusual spatial and temporal patterns of C deaminations by AID observed in vitro (*47*, *48*). As shown in Figure 2b, C deaminations occur in dense clusters, of approximately 3−10 bases, which are interdispersed between nondeaminated regions on ssDNA. The clusters are distributed randomly with WRC hot spots deaminated first followed at later times by deamination of neighboring NNC neutral and SYC cold spots (*48*). This type of deamination pattern could be generated if each AID monomer were to bind randomly to the same ssDNA strand and catalyze localized deaminations in a processive manner. While one of the subunits remains bound, the other could dissociate and move to a distal location on the same ssDNA strand, and this process could then be repeated multiple times to generate clusters of deaminated C moieties, with different ssDNA substrates having different clustering patterns (Figure 2b).

Biochemical Pathways on the Road to SHM and CSR

AID and transcription work hand in hand to initiate SHM and CSR, but the two processes diverge thereafter. Thus, there are AID mutations that affect CSR but not SHM and visa versa (*50*, *61*, *62*), and there are B cells expressing IgM antibodies with hypermutating V regions, and B cells expressing IgA and IgG antibodies without SHM (*15*). A plausible, albeit tentative, emerging model depicts AID acting at exposed C sites on single-stranded DNA during transcription (Figure 4). SHM requires Ig V gene transcription (*51*, *52*), while CSR depends on switch (S) region transcription (*71*). Both pathways evolve from G·U mispairs using markedly different mechanisms.

SHM is characterized by a variety of V gene base substitutions distributed approximately equally on both DNA strands. Nontranscribed strand C → T mutations can result by copying U with a high-fidelity DNA polymerase (Figure 4). Transcribed strand mutations can occur following the removal of U on the nontranscribed strand during BER (patch repair), or by the processing of U·G mispairs during MMR (Figure 4). Both types of repair cause gapped DNA structures that can expose the transcribed strand to AID and to error-prone DNA polymerases. AID can initiate C → T mutations on the transcribed strand, while error-prone polymerases can generate all types of mutations, including a secondary hot spot mutation in WA motifs favored by pol η (*42*). Xeroderma pigmentosum patients lacking pol η seem to have a normal immune response; their V genes contain elevated levels of mutations at G−C sites, but have depressed levels of mutations at A−T sites in accord with the absence of pol η (*55*, *72*).

CSR requires transcription of a targeted S region that produces sterile (i.e., nontranslated) germ-line transcripts (*45*). Biochemical studies of CSR have shown that transcribed S regions adopt triple-strand R-loop structures (*44*). The transcribed strand forms a stable RNA−DNA hybrid with the transcript, while the G-rich nontranscribed strand remains single-stranded (*73*). The R-loops appear to vary in length from 150 to 500 bp and may be stabilized using G4 DNA tetrads (*74*). However, R-loop structures may not be a requirement for CSR based on the recent finding that the Sμ region in *Xenopus laevis*, which is A−T rich and not prone to forming R-loops, can functionally replace a mouse switch region to mediate CSR in vivo (*75*). Actively transcribed mouse and *Xenopus* Sμ DNA is efficiently deaminated by AID in the presence of RPA (*49*, *75*).

Studies with cell-free systems reinforce the idea that AID can operate effectively during transcription. Transcription-dependent AID-catalyzed deaminations in vitro favor the exposed nontranscribed strand (*34*, *47*−*49*, *53*). When T7 RNA polymerase was used to transcribe *lacZ* on closed circular dsDNA under control of the T7 promoter, the spectrum and distribution of AID-catalyzed deaminations were similar to those observed on naked ssDNA exposed to AID (*48*). Thus, while working within the confines of a moving transcription bubble, AID retains specificity for WRC hot spot motifs and continues to form deamination clusters, primarily on the nontranscribed strand. At present, insofar as the observations that AID acting during transcription appears to simulate several hallmark features of SHM and CSR, it seems fair to say that recent in vitro studies with AID deaminating ssDNA represent a promising start toward a developing a more complete biochemical reconstitution of Ab diversification. But the story has just begun, as numerous complex events occurring downstream from the initial C targeting action of AID remain to be addressed.

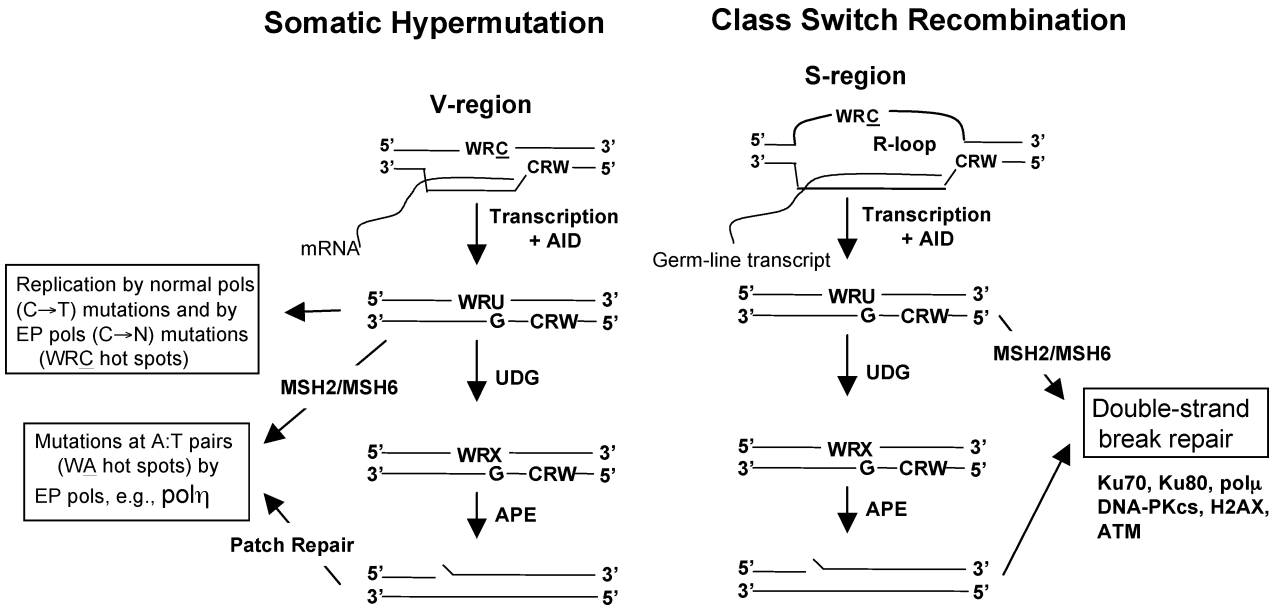

FIGURE 4: AID-initiated SHM and CSR putative biochemical pathways. AID acting on ssDNA exposed in the transcription bubble in the V region (or R-loop formed by transcription of the S region) deaminates C in the WRC hot-spot sequences to initiate SHM and CSR. Subsequent copying of U by a high-fidelity DNA polymerase will likely result in a C → T transition mutation, or copying of U by an error-prone (EP) polymerase could generate C → N transition and transversion mutations. A second stage of SHM diversification leading to mutations at A:T base pairs depends on the actions of uracil glycosylase (UNG) and postreplication mismatch repair (MMR) proteins MSH2 and MSH6. Mutations at WA hot spots are probably made by pol η during MMR and BER patch repair synthesis. Processing of G•U mismatches during CSR in mice is also dependent on UDG and MSH2/MSH6. Ku70, Ku80, pol μ, DNA-PKcs, H2AX, and ATM proteins that are important for nonhomologous end joining reactions may be involved in double-strand break repair during CSR. X on the DNA denotes an abasic site.

Besides its initiating role for SHM and CSR, AID was shown to recruit DNA-PKcs to DNA, which were required for repairing double-stranded breaks during CSR. C-Terminal deletion AID mutants with an impaired interaction with DNA-PKcs cause the death of cells undergoing CSR upon stimulation due to accumulation of unrepaired double-stranded breaks (*76*).

As a reflection on an important open issue in CSR, how is AID selectively recruited to certain S regions while avoiding other S regions in response to stimulation with mitogens and cytokines? Recently, Scharff and colleagues have shown that RNA pol II distribution and histone H3 hyperacetylation correlate with the transcriptional activity of selected S regions in response to stimuli (*77*). They suggested that the increase in density of RNA pol II at certain S regions after stimulation could lead to preferential recruitment of AID to the sites, in accord with the observaton that RNA pol II and AID co-immunoprecipitate in cells undergoing CSR (*46*). To what extent might RPA or transcription factors determine AID access to transcribed S regions (*49*)? The same questions apply to SHM: for example, how is AID recruited to transcribed V genes? What keeps AID from deaminating transcribed C genes?

Biochemical events involving MMR and BER downstream of AID-targeted deamination are likewise opaque. Genetic studies in mice deficient in UNG and MSH2/MSH6 show that BER and MMR are involved in processing of G•U mismatches (*78*−*80*) (Figure 4). UDG deficiencies in mice and humans skew SHM mutation spectra toward C → T transitions and reduce CSR by ∼5−10-fold (*81*, *82*). Although there are a number of other mammalian glycosylases, e.g., SMUG1, TDG, and MBD4, genetic data show that only UDG is required for SHM and CSR (*80*, *83*). Not surprisingly, MMR and BER appear to have partially overlapping functions. MSH2- and MHS6-deficient (but not MSH3-deficient) mice show reduced levels of isotype switching and SHM mutations at A•T base pairs (*78*, *79*, *84*, *85*). Mice deficient in both UDG and MSH2 no longer perform CSR, and IgV hypermutation no longer occurs at A•T base pairs (*80*). These results suggest that uracil excision by UDG is the major pathway for processing G•U mismatches during CSR, with MSH2/MSH6 proteins providing a backup pathway (*80*) (Figure 4). Other proteins implicated in CSR include Ku70, Ku80 and DNA-PKcs, and pol μ (reviewed in refs *15*, *23*, and *45*). H2AX- and ATM-deficient mice also exhibit impaired CSR activity (*45*, *86*, *87*). It is likely that both UDG and MMR proteins initiate CSR and additional mutations at A−T sites during SHM (*15*).

INNATE RESISTANCE AGAINST RETROVIRUSES BY APOBEC3G AND APOBEC3F

The DNA deaminases Apobec3G and Apobec3F participate in a variety of defense mechanisms directed specifically against retroviruses. Deamination occurring on DNA generated by reverse transcription of the viral RNA results in the conversion of ∼1−2% of all C residues to U (*17*). The presence of large numbers of U residues presumably causes minus strand degradation by the action of UDG and APE, or perhaps results in viral death by introducing catastrophic numbers of errors in essential genes.

HIV-1 encodes Vif (virus infectivity factor), which is a small protein that allows viral replication in nonpermissive cells. For example, Vif-defective HIV-1 fails to replicate in either H9 or CEM15 cultured cells. In contrast, Vif is not required for viral infection of permissive cells such as HeLa, COS, Jurkat, or 293 T (reviewed in ref *17*). The nonpermissive phenotype is dominant, suggesting the presence of

at least one inhibitory factor that acts to repress replication of Vif-deficient virions in nonpermissive cells (*88*, *89*). A subtractive mRNA screen revealed the presence of that inhibitory factor, Apobec3G (CEM15) (*90*).

Apobec3G exhibits robust expression in T lymphocytes and in macrophages, both of which are main targets for HIV infection. It modifies the HIV genome by deaminating C preferentially at 5′ CC motifs generating large numbers of G → A mutations (*91*−*94*). Biochemical data show that Apobec3G can deaminate C on ssDNA (*84*, *108*, *109*). Apobec3G is encased in Vif-defective HIV particles. Upon infection of naïve T cells, Apobec3G deaminates C on nascent DNA minus strands generated by reverse transcription of the retroviral RNA. When Vif is present, however, the formation of a Vif−Apobec3G complex prevents the incorporation of Apobec3G into the virion, and instead promotes its degradation through a proteasome-dependent pathway (*95*−*103*). Simian immunodeficiency (SIV), murine leukemia virus (MLV), and equine infectious anemia virus (EIAV) are examples of other retroviral genomes that are attacked by Apobec3G (*91*, *92*).

Another Apobec3 homologue, Apobec3F, suppresses HIV-1 replication, which is analogous to the action of Apobec3G, and is also inhibited by Vif protein (*104*−*106*). These two Apobec homologues coexpress and form active heterodimers, or perhaps higher-order multimers in vivo in nonpermissive human cells (*104*). It is possible that several different interacting deaminase homologues working in tandem are able to trigger the destruction of viral cDNA more efficiently than each working alone. Other Apobec homologues, including human Apobec3B, Apobec3C, rat Apobec1, and mouse Apobec3, have been shown to have antiretroviral activity in cultured cells (*107*).

The interaction of Vif with Apobec3G is species specific (*97*). Vif from HIV-1 and SIV neutralize Apobec3G from the host species, but generally not from other species. HIV-1 Vif blocks human Apobec3G but does not act on the homologous African green monkey (AGM) deaminase, whereas the reverse is observed with SIV Vif from AGM; namely, SIV Vif blocks Apobec3G from AGM but does not affect human Apobec3G. An amino acid at position 128 of Apobec3G (Asp in the human form and Lys in AGM) appears to determine the species-specific interactions with Vif (*108*−*110*). Human Apobec3B and Apobec3C can also efficiently inhibit SIV (*111*).

The antiviral specificity of Apobec3G extends beyond retroviruses, as it has also been shown to block hepatitis B virus (HBV) (*112*, *113*). Human Apobec3G also inhibits MLV, whereas rodent Apobec1 and Apobec3 do not affect MLV infectivity. At present, however, no Vif-like protein is known to be encoded by MLV. Thus, it remains to be determined if the antiretroviral species specificities for the DNA deaminase homologues are defined principally by their interactions with viral proteins having properties analogous to those of HIV-1-encoded Vif (*107*).

DNA DEAMINASES AND GENOMIC INSTABILITY

In humans, C → T mutations are thought to occur spontaneously in both normal and tumor cells, with a significant fraction occurring in CpG sequences caused by deamination of 5MeC (*6*). It is possible, however, that a non-negligible fraction of C → T mutations might also be caused by Apobec DNA deaminases in normal and diseased tissues. Although the expression of AID seems to be restricted to lymphoid cells, substantial quantities of Apobec3G and -3F mRNA have been observed in lung, liver, kidney, and pancreas, in addition to lymphoid tissue (*104*). The distribution of the other Apobec homologues is not currently known. A low level of expression of one or more of the DNA deaminases might have a large impact on deamination of C, especially in at risk sequences corresponding to hot spot motifs.

DNA Deaminases and Cancer. Cellular expression of DNA deaminases and their mutations must be limited to the "right place" and the "right time". Thus, the transcription of AID is tightly regulated during normal B cell development. With respect to place, AID expression is restricted to centroblast cells within germinal centers, while with respect to time, mRNA synthesis occurs ∼2 days after in vitro stimulation and sharply declines by the fourth day (*24*, *114*, *115*). A significant elevation in mutation and genomic instability is likely to occur when AID is expressed gratuitously, and this can lead to cancer.

Aberrant SHM has been observed in ∼50% of diffuse large B cell lymphomas, in ∼20% of AIDS-related non-Hodgkin's lymphomas, and in a sizable fraction of primary central nervous system lymphomas derived from immunocompetent HIV-negative patients (*116*−*118*). Consistent with the inappropriate action of AID, B cell lymphomas contain numerous mutations in WRC/GYW hot spots in the 5′ regions of B cell protooncogenes Bcl-6, PIM1, PAX5, RhoH/TTF, and cMYC, and in accord with Ig SHM, the mutations tend to occur in a 2 kb region from the end of promoters. B cell lymphoma can be caused by a chronic viral infection. For instance, hepatitis C virus (HCV), which causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, has been detected in ∼30% of patients with malignant B cell non-Hodgkin's lymphoma (*119*). Recently, it has been shown that both acute and chronic infection by HCV lead to the enhancement of mutations in Ig genes and proto-oncogenes through induction of AID and error-prone DNA polymerases (*120*). Similarly, EBV membrane proteins can induce AID expression (*121*). Point mutations at deaminated C sites may represent just the tip of the iceberg, because the unregulated action of AID can cause Ig chromosomal translocations frequently associated with mature B cell malignancies in humans. In mice, C-myc/Ig chromosomal translocations are known to depend on AID expression (*122*). Recently, however, it was reported that AID does not contribute to the generation of initial *Myc*-S region translocations, further suggesting that AID might be acting downstream of S region double-strand breaks (*123*). Perhaps there are two distinct pathways for DSB initiation in S regions.

The inability to regulate AID expression is also responsible for tumorigenesis in mice. Transgenic mice with constitutive expression of AID tend to develop malignant T cell lymphoma, micro-adenomas, and lung adenocarcinomas (*124*). Here one observes an increase in the frequency of point mutations, but not translocations, in T cell receptors and *c-myc* genes from T cell lymphomas (*124*). The distribution and specificity of mutations are similar to those observed in B cell lymphomas (*116*) and in cultured cells that overexpress AID (*27*, *28*, *125*), which suggests that AID is responsible

for tumorigenesis. In principle, however, any or all of the DNA deaminases have the potential to create similar havoc. Expression of Apobec1 in rabbit and mouse liver correlates with the appearance of hepatocellular carcinomas in transgenic animals (*126*).

Participation of Antiviral DNA Deaminases in Controlling Endogenous Retroelements. The wider-ranging expression pattern of Apobec3G and -3F correlates with their ability to induce G → A mutations in HBV and thereby restrict infectivity by the retroelement. It is likely therefore that DNA deaminases play a similar role in controlling endogenous retroelements (*20*), such as endogeneous retroviruses and non-long terminal repeat (non-LTP) retrotransposons. The non-LTP retrotransposons account for ~27% of the mass of the human genome and are likely to influence DNA structure and function (*127*). Retrotransposons replicate using reverse transcriptase to copy an RNA transcript into genomic DNA. Transpositions caused by the retroelements, such as active transposons of the Ta family, result in a variety of genome rearrangements and polymorphisms (*128*). Insertion mutations caused by retrotranspositions are associated with muscular dystrophy, hemophilia, and breast cancer (*129*). On the basis of the ability of Apobec homologues to target reverse transcripts for deamination at favored C motifs, the time is ripe to address the possible contribution of antiviral DNA deaminases to genome destabilization through retrotransposon activation mechanisms. Although Trono and colleagues (*130*) have just reported that APOBEC3G does not affect human LINE-1 retrotransposition in a cell culture assay, other Apobec homologues might play some role in this process.

Aberrant Deamination of At Risk Sequences. Exposed ssDNA regions, occurring, for example, in genes undergoing active transcription, are likely targets for DNA deaminases. DNA deaminases may attack the nontranscribed strand (*34*, *47*−*49*, *53*) and may also gain access to regions of supercoiled dsDNA situated adjacent to a moving transcription bubble (*131*, *132*). Favored mutational targets include WRC for AID, YCC for Apobec3G, and TC for Apobec1 and Apobec3F. Preferred targets for other Apobec homologues are listed in Table 1.

At risk motifs are likely to be tissue-specific, depending on which of the DNA deaminases are present. To illustrate this point, an analysis of more than 200 point mutations in proto-oncogenes in several types of B cell tumors revealed that most of the mutations (82%) are at C•G base pairs, and a majority of these (64%) occur in WRC motifs (*133*). The mutational spectra are consistent with the "inappropriate" action of AID in B cell tumors.

However, WRC is not the preferred mutational target for C → T mutations in tumor suppressor genes p53 and APC in different types of tumors. Elevated mutations in APC were found at 5′ TC sites in colorectal cancer, which is consistent with the action of Apobec1 acting on ssDNA in *E. coli* and in vitro; notably, Apobec1 is highly expressed in colorectal tissue (*133*). Apobec3F also targets 5′ TC sites, although its presence in colorectal tissue has not been established. Since it has been shown that AID deaminates 5MeC residues with an efficiency of roughly 10−30% compared to C in vitro (*31*, *35*, *36*), it is possible that AID and other Apobec homologues can attack MeCpG sequences in vivo, which may result in biological havoc. The classic example of an at risk sequence is methylated C in CpG dinucleotides, where C → T mutations represent a disproportionately large fraction of mutations in human disease. It has been estimated that these comprise approximately one-quarter of p53 tumor suppressor mutations (*134*).

Secondary structures occurring in double-stranded nucleic acids may also be susceptible to deamination at C sites. Sequences prone to assuming non-W−C structures that have been implicated in human genetic disease include triplexes, left-handed Z DNA, G4 DNA tetrads, and slipped and sticky DNA structures, including trinucleotide repeats implicated in approximately a dozen neurodegenerative diseases (*135*). DNA deaminases could in principle act on these types of secondary structures to initiate dsDNA breaks leading to DNA rearrangements.

The Ig switch region offers a concrete example of a sequence that assumes a secondary structure to serve as a substrate for AID. The Ig switch sequence provides a biologically beneficial substrate allowing AID to initiate CSR. DNA deaminases might contribute substantially to the mutational load by attacking similar types of substrates with regions containing DNA secondary structure. It is important to investigate how Apobec enzymes might be responsible for activation of oncogenes and inactivation of tumor suppressor genes and more generally cause mutations leading to human disease. From a biological perspective, it is important to re-examine the origins of C → T mutations, what fraction is caused by spontaneous deamination of C, and what fraction arises from the inappropriate action of DNA deaminases.

TOWARD A BIOCHEMICAL UNDERSTANDING OF IMMUNITY AND DISEASE WITH AID

It is now clear that AID is a common catalytic intermediate in the branched pathway leading to SHM and CSR, initiating both processes by the targeted C → U conversion (Figure 4). To understand how the succeeding biochemical steps are organized, it will be necessary to include the relevant DNA synthesis and repair proteins in a reconstituted cell-free system. This type of biochemical approach to investigating links among replication, transcription, and repair should prove to be instrumental in deciphering the complexities of SHM and CSR. A promising first step has been taken by showing that AID acting alone on naked ssDNA or working in conjunction with T7 RNA polymerase on transcribed DNA reflects several important aspects of what happens in vivo. Embodied in the SHM−CSR model sketch shown in Figure 4 are a number of biochemical challenges, some tractable and others much less so. The good news is that cell-free systems are currently available that mimic prominent features of human replication, transcription, BER, MMR, and NHEJ, all of which are likely to be involved in SHM, CSR, or both. The trick will be to include AID in the mix to determine how targeting occurs in Ig genes but not in all highly expressed genes in centroblast B cells, why both strands in vivo are equally targeted for mutations, and why certain non-Ig genes but not others are targeted in different B cell lymphomas. These are just a few of the unresolved biochemical questions that one can now begin to address with AID in hand. Although the devil is in the details, the prospects seem promising. When properly targeted, C

deaminations initiate favorable mutations that trigger Ig diversification and protection against viral infection. For the Apobec family of nucleic acid cytidine deaminases, it is imperative to investigate both sides of the mutational coin, the biochemical basis of immunity when things go right and the incidence of disease when things go wrong.

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