Review

Post-translational regulation of activation-induced cytidine deaminase

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The assembled *immunoglobulin* genes in the B cells of mice and humans are altered by distinct processes known as class switch recombination (CSR) and somatic hypermutation, leading to diversification of the antibody repertoire. These two DNA modification processes are initiated by the B cell-specific protein factor activation-induced cytidine deaminase (AID). AID is post-translationally modified by phosphorylation at multiple sites, although functional significance during CSR has been implicated only for phosphorylation at serine-38 (S38). Although multiple laboratories have demonstrated that AID function is regulated via phosphorylation at S38, the precise biological role of S38 phosphorylation has been a topic of debate. Here, we discuss our interpretation of the significance of AID regulation via phosphorylation and also discuss how this form of AID regulation may have evolved in higher organisms.

Keywords: activation-induced cytidine deaminase; class switch recombination; evolution; phosphorylation; somatic hypermutation

1. INTRODUCTION

The DNA sequences that encode the variable regions of immunoglobulin (Ig) heavy (H) and light (L) chains are assembled from variable (V), diversity (D) and joining (J) DNA segments during early B cell development (Dudley *et al.* 2005). The products of recombination activating genes 1 and 2 are expressed in a lymphocyte-specific manner and initiate V(D)J recombination by recognizing and introducing DNA double-strand breaks (DSBs) at the recombination signal sequences that flank germ line V, D and J segments. Subsequently, non-homologous end-joining fuses broken V, D and J segments (Dudley *et al.* 2005). Productive assembly of an IgH variable region exon places a VDJ exon upstream of the C μ IgH constant region exons, allowing generation of μ heavy chain (HC) mRNA and μ HC proteins. The μ HC pairs with IgL chains produced from a functionally rearranged IgL locus to form IgM, leading to generation of surface IgM⁺B cells. IgM⁺B cells migrate to secondary lymphoid organs where, upon encounter with cognate antigens, they are activated to undergo class switch recombination (CSR) and somatic hypermutation (SHM). CSR is a recombination/deletion process that replaces C μ with a different set of IgH constant region exons (Chaudhuri *et al.* 2007) and SHM introduces point mutations into variable region exons

(Di Noia & Neuberger 2007). Both CSR and SHM in mice and humans are initiated by activation-induced (cytidine) deaminase (AID; Muramatsu *et al.* 2000).

2. CLASS SWITCH RECOMBINATION

The Ig HC constant (C_H) region determines biological effector functions of Ig molecules. In mice there are eight sets of C_H exons ('C_H genes') organized as 5'-VDJ-C μ -C δ -C γ 3-C γ 1-C γ 2b-C γ 2a-C ϵ -C α -3'. Mice produce five IgH classes, IgM, IgD, IgG, IgE and IgA, encoded by μ , δ , γ , and α C_H genes, respectively. Each C_H region is specialized in determining how an Ig molecule eliminates antigen. CSR allows the assembled VDJ exon, initially expressed with the C μ exons, to be expressed with one of the sets of downstream C_H exons (Chaudhuri *et al.* 2007), and hence enables the production of different IgH classes. CSR occurs within large, repetitive switch (S) regions located 5' of each set of C_H exons. CSR is a deletional recombination event that occurs via the introduction of DSBs into the donor S μ and a downstream acceptor S region, followed by joining of broken donor and acceptor S regions to each other (Honjo *et al.* 2002).

Mammalian S regions are 1–10 kb in length, unusually G-rich on the template (transcribed) strand, and primarily composed of tandem repetitive sequences within which certain motifs predominate (Chaudhuri & Alt 2004). S μ is exceptionally repetitive and enriched in GAGCT motifs, with the AGCT palindrome representing a canonical RGYW motif (where R=purine, Y=pyrimidine and W=A or T). The RGYW motif and the reciprocal WRCY motif are

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SHM hotspots (see later). $\text{S}\gamma 1$ is the largest S region and, like other $\text{S}\gamma$ sequences, carries multiple 49 bp repeats that are rich in RGYW motifs (Chaudhuri & Alt 2004). In some species, S regions are quite divergent; *Xenopus* S regions are AT-rich (Du Pasquier *et al.* 2000). The vast majority of CSR junctions occur within or, sometimes, just beyond S regions (Dunnick *et al.* 1993). In normal B cells, deletion of the $\text{S}\mu$ repeats greatly reduced CSR (Luby *et al.* 2001; Khamlichi *et al.* 2004) and deletion of the 10 kb $\text{S}\gamma 1$ abrogated CSR to $\text{C}\gamma 1$ (Shinkura *et al.* 2003). Thus, S regions appear to be the main CSR targets.

The different C_H exons are organized into units in which germ line transcription initiates from a promoter lying upstream of individual S regions (Chaudhuri *et al.* 2007). Germ line transcription is a prerequisite for CSR (Manis *et al.* 2002). *In vivo*, CSR is stimulated by T cell-dependent and T cell-independent antigens, which can be mimicked *in vitro* by activating B cells with anti-CD40 or bacterial lipopolysaccharide in the presence of cytokines such as interleukin-4 (IL-4; Chaudhuri & Alt 2004). Different activators and cytokine combinations direct CSR to particular S regions by stimulating germ line transcription at the promoter upstream of particular target S regions (Manis *et al.* 2002; Chaudhuri *et al.* 2007).

3. SOMATIC HYPERMUTATION

SHM introduces point mutations, and sometimes small insertions or deletions, at a very high rate (estimated to be approx. 10^{-3} – 10^{-4} /base pair/generation) into the variable region exons of IgH and IgL genes, allowing selection of B cells with increased affinity for antigen (Li *et al.* 2004). SHM occurs preferentially around RGYW and WRCY motifs (Rogozin & Kolchanov 1992), which are particularly abundant in the DNA sequences that encode the variable region complementarity-determining regions (Wagner *et al.* 1995), the site of the majority of the antigen contact residues. SHM requires transcription (Fukita *et al.* 1998), with mutations occurring from 100–200 bp from the promoter and extending 1.5–2 kb downstream, generally sparing C_H exons (Longerich *et al.* 2006). In transgenic SHM substrate studies, variable region exons or their promoters can be replaced with other sequences or non-Ig promoters with varying degrees of SHM (Storb *et al.* 2001; Odegard & Schatz 2006), indicating variable region exons are not unique with respect to SHM targeting. SHM can also occur to varying degrees at non-Ig genes, although at much lower levels (Odegard & Schatz 2006; Liu *et al.* 2008). Precise sequence requirements and *cis*-acting elements that target SHM *in vivo* remain to be defined (Odegard & Schatz 2006).

4. ROLE OF ACTIVATION-INDUCED CYTIDINE DEAMINASE IN CLASS SWITCH RECOMBINATION AND SOMATIC HYPERMUTATION

AID is a 24 kDa protein that is expressed predominantly in activated B cells and is absolutely required for CSR and SHM in mice and humans (Muramatsu *et al.* 2000; Revy *et al.* 2000). While the role of AID in CSR

and SHM *in vivo* has been debated, it has become clear that AID functions in these processes by the deamination of cytosine residues in S regions and variable region exons to uracil (Chaudhuri *et al.* 2007). Genetic studies indicate that AID-generated uracil residues in S regions and variable region exons are processed by base excision repair (BER) and mismatch repair (MMR) to generate DSBs and point mutations, respectively (Di Noia & Neuberger 2007). Other evidence in support of the DNA deamination model include the ability of AID to mutate DNA in bacteria (Petersen-Mahrt *et al.* 2002); the biochemical demonstration that purified B cell AID (Chaudhuri *et al.* 2003) and recombinant AID (Bransteitter *et al.* 2003; Dickerson *et al.* 2003; Ramiro *et al.* 2003; Sohail *et al.* 2003) have robust single-stranded DNA (ssDNA)-specific cytidine deaminase activity; and the demonstration that AID binds, in a CSR-dependent fashion, to S regions in activated B cells (Nambu *et al.* 2003; Chaudhuri *et al.* 2004). In addition, AID preferentially deaminates cytosine residues within WRC sequences *in vitro* (Pham *et al.* 2003). While AID deaminates ssDNA substrates *in vitro*, it does not deaminate double-stranded DNA (dsDNA) substrates (Chaudhuri *et al.* 2003). Several mechanisms have been implicated in AID access to duplex V(D) J exons and S regions *in vivo* as discussed in the following paragraphs.

Owing to their high G/C content and G richness on the non-template strand, mammalian S regions generate ssDNA within R-loops transcribed in the physiologic direction (Tian & Alt 2000; Yu *et al.* 2003), suggesting that transcription might allow AID to access ssDNA substrates by this mechanism, a notion supported by finding that optimal S region function depends on their transcriptional orientation *in vivo* (Shinkura *et al.* 2003). Moreover, *in vitro* purified AID deaminates the non-template strand of T7 RNA polymerase-transcribed dsDNA sequences that form R-loops (e.g. S regions in sense orientation) but not transcribed dsDNA sequences that do not form R-loops (e.g. V(D)J exons; Chaudhuri *et al.* 2003). Thus, R-loop forming ability may have evolved in mammalian S regions to enhance AID access during CSR.

In vitro assays to identify factors that allow purified B-cell AID to deaminate transcribed SHM substrates (do not form R-loops) revealed that replication protein A (RPA), a trimeric ssDNA-binding protein involved in replication and repair, serves as a cofactor to promote efficient AID deamination of non-R-loop forming dsDNA 'SHM substrates' that contain AGCT motifs; (a canonical RGYW motif; Chaudhuri *et al.* 2004). The 32 kDa subunit of RPA interacts with purified B-cell AID, and the AID/RPA complex binds to transcribed WRCY/RGYW-containing DNA *in vitro*, hence leading to deamination within or near SHM motifs (Chaudhuri *et al.* 2004). The AID/RPA complex may bind to and stabilize ssDNA within transcription bubbles to allow AID to access ssDNA substrates (Chaudhuri *et al.* 2004).

Both AID and Ig gene SHM are present in bony fish. However, CSR first occurs evolutionarily in amphibians, suggesting that CSR evolved after SHM (Stavnezer & Amemiya 2004). Notably, *Xenopus* $\text{S}\mu$ (XS μ) is A/T rich and does not form R-loops when transcribed *in vitro* (Zarrin *et al.* 2004). Yet, XS μ can

replace mouse S γ 1 to promote substantial CSR in mouse B cells (Zarrin *et al.* 2004). CSR junctions within XS μ in mice occurred in a region of densely packed AGCT sequences in the 5' portion of XS μ ; when this sequence was inverted *in vivo*, CSR junctions tracked with the AGCT motifs (Zarrin *et al.* 2004). Moreover, the AGCT repeat region was the predominant site of deamination by AID/RPA *in vitro*, regardless of orientation (Zarrin *et al.* 2004). Overall, these findings support the notion that the CSR activities of AID evolved from SHM functions (Barreto *et al.* 2005; Wakae *et al.* 2006), the latter of which may, at least in part, employ the ability of the AID/RPA complex or related mechanisms to access transcribed sequences rich in SHM motifs (Chaudhuri *et al.* 2004; Zarrin *et al.* 2004). Various findings, including the fact that S regions are rich in WRCY/RGYW motifs (particularly AGCT motifs), suggested the possibility that the AID/RPA mode of targeting also may play a role in mammalian CSR (Chaudhuri *et al.* 2004).

5. POST-TRANSLATIONAL MODIFICATION OF ACTIVATION-INDUCED CYTIDINE DEAMINASE

The question of how AID preferentially targets certain regions within Ig loci and not many other genomic sequences remains unanswered, although transcriptional regulation and target motif density may be contributing factors (Longerich *et al.* 2006). Other intriguing questions are why AID elicits point mutations in V(D)J exons and DSBs (in addition to point mutations) in S regions, as well as how B cells can differentially activate CSR versus SHM within narrow regions of the IgH locus. The density of target motifs is one simple explanation for DSB versus SHM activity, but this remains to be proven experimentally (Chaudhuri *et al.* 2007). Differential transcription of target loci directs AID to different S regions during CSR (Chaudhuri *et al.* 2007) and might similarly help target AID to variable region exons during SHM versus adjacent S regions during CSR. Another potential AID regulatory mechanism might involve the channelling of deaminated cytidines into normal versus abnormal BER or MMR processing (Di Noia & Neuberger 2007). In addition, assays of ectopically expressed mutant forms of AID suggested that particular AID domains might interact with specific cofactors or repair factors to differentially effect SHM or CSR (Barreto *et al.* 2003; Ta *et al.* 2003; Shinkura *et al.* 2004; Imai *et al.* 2005), potentially in the context of different AID post-transcriptional modifications.

Post-translational modifications influence AID activity *in vitro*. The ability of AID isolated from activated murine B cells to interact with RPA and function in the transcription-dependent dsDNA deamination assay is dependent on phosphorylation at Serine-38 (S38; Basu *et al.* 2005). AID S38 lies within a cAMP-dependent protein kinase A (PKA) consensus motif and, correspondingly, AID can be phosphorylated *in vitro* and *in vivo* at S38 by PKA (Basu *et al.* 2005; Pasqualucci *et al.* 2006). PKA-phosphorylated AID gains the ability to bind RPA and mediate dsDNA deamination of transcribed SHM substrates (Basu *et al.* 2005). Mutation of AID S38 to alanine (AID^{S38A})

sparcs ssDNA deamination activity, but markedly decreases PKA phosphorylation, and RPA interaction and function in transcription-dependent dsDNA deamination assays (Basu *et al.* 2005). Retrovirally expressed AID^{S38A} had reduced CSR activity (15–30% of WT activity) in activated B cells (Basu *et al.* 2005; McBride *et al.* 2006; Pasqualucci *et al.* 2006). Likewise, PKA inhibition decreased CSR *in vivo*, while deletion of the PKA-negative regulatory subunit increased CSR *in vivo* (Pasqualucci *et al.* 2006). Thus, current studies support the proposal that AID phosphorylation at S38 is one mechanism for augmenting the ability of AID to initiate CSR, supporting R-loop-independent means for accessing transcribed S regions (Basu *et al.* 2005). In addition, retrovirally expressed AID^{S38A} had reduced capacity to mediate SHM in both mouse and chicken DT40 cells (McBride *et al.* 2006; Chatterji *et al.* 2007). Notably, however, zebrafish AID (zAID) lacks an S38-equivalent PKA phosphorylation site (figure 1); yet, zAID can rescue CSR in AID-deficient mouse B cells (Barreto *et al.* 2005; Wakae *et al.* 2006). Superficially, the latter finding may be viewed as a challenge to the notion that S38 phosphorylation has a physiological function in the regulation of AID activity (discussed below; Shinkura *et al.* 2007; Pham *et al.* 2008).

6. PHYSIOLOGICAL RELEVANCE OF PHOSPHORYLATION

The potential role of AID phosphorylation at S38 has been controversial. Thus, work from the Honjo laboratory questioned whether AID was significantly phosphorylated (potentially due to insensitivity of the assay used) and further concluded, in contrast to our findings and those of others (Basu *et al.* 2005; McBride *et al.* 2006; Pasqualucci *et al.* 2006), that an AID^{S38A} retroviral construct supported CSR at nearly WT levels (Shinkura *et al.* 2007). We addressed this apparent controversy by comparing CSR activity and expression of AID^{WT} with the corresponding AID^{S38A} constructs generated in our laboratory as well as with AID^{WT} and AID^{S38A} constructs from Shinkura and Honjo (Basu *et al.* 2007). We found that the Shinkura/Honjo AID^{WT} construct had similar CSR activity to our AID^{WT} construct, but that the Shinkura/Honjo AID^{S38A} mutant, our AID^{S38A} mutant, had substantially reduced capacity to support CSR (Basu *et al.* 2007). While the Shinkura/Honjo AID^{S38A} construct did support a somewhat higher level of CSR (35% of WT) than ours (15% of WT), we argued that the difference probably reflected differences in expression levels of the two constructs (Basu *et al.* 2007). In this regard, the AID^{S38A} mutant retains significant residual CSR activity (Basu *et al.* 2005), which most likely reflects AID access to S regions via mechanisms that do not depend on S38 phosphorylation (e.g. R-loops; Chaudhuri *et al.* 2003; Basu *et al.* 2005, 2007). Thus, assaying expression of such hypomorphic AID mutants under conditions in which AID^{WT} activity is saturated might lead to significant differences in the measurement of hypomorph activity in the context of differences in expression levels and, if over-expressed enough, apparent levels might approach those of WT

kgrretylcyvvkrrd s atsfsl	<i>Homo sapiens</i>
kgrretylcyvvkrrd s atsfsl	<i>Pan troglodytes</i>
kgrhetylcyvvkrrd s atscsl	<i>Mus musculus</i>
kgrhetylcyivkrry s svscal	<i>Xenopus laevis</i>
kgrretylcyvvkrrd s atscsl	<i>Gallus gallus</i>
rgrhetylcfvvkrrigp d slsf	<i>Danio rerio</i>
rgrhetylcfvvkrrvgp d tltf	<i>Takifugu rubripes</i>
rgrnetylcfvvkrrnsp d slsf	<i>Ictalurus punctatus</i>

Figure 1. AID amino acid multiple alignment for various species corresponding to the region around mouse AID position 38. AID Serine-38 (s), along with the protein kinase A consensus motif (RRXS), is present in all organisms that undergo SHM as well as CSR, whereas the aspartate D44 residue (d) in teleost zebrafish is present only in those organisms that undergo SHM. *Homo sapiens* (human), *Pan troglodytes* (chimpanzee), *Mus musculus* (mouse), *Xenopus laevis* (frog), *Gallus gallus* (chicken), *Danio rerio* (zebrafish), *Takifugu rubripes* (fugu), *Ictalurus punctatus* (catfish).

(Basu *et al.* 2007). Recently, we have generated mouse B cells that harbour the homozygous S38A mutation in their genomic DNA and, based on preliminary analyses, found that they also had substantially impaired ability to carry out IgH CSR (H.-L. Cheng, B. Vuong, F. W. Alt & J. Chaudhuri 2008, unpublished data).

Another apparently contradictory finding was made by Goodman and colleagues, who recently examined the ability of baculovirus-expressed AID to deaminate an *in vitro*-transcribed dsDNA substrate and concluded that neither phosphorylation nor RPA was required for AID activity (Pham *et al.* 2008). Notably, however, inspection of the substrate employed in these studies revealed 45 per cent G residues on the non-template strand, which strongly suggests the potential for the substrate to form R-loops upon transcription. Therefore, this observation may well be in accord with our prior studies in which we had reported that purified AID from mammalian cells could robustly deaminate R-loop containing substrates in the absence of phosphorylation or RPA (Chaudhuri *et al.* 2003, 2004).

7. EVOLUTIONARY DIFFERENCES IN REGULATION OF ACTIVATION-INDUCED CYTIDINE DEAMINASE VIA PHOSPHORYLATION

Zebrafish and other bony fish possess AID and undergo SHM but do not undergo CSR. Yet zAID can replace function in AID-deficient mouse B cells to catalyse substantial CSR (Barreto *et al.* 2005; Wakae *et al.* 2006). Thus, it seems likely that lack of CSR in zebrafish reflects lack of different C_H genes and S regions (Chaudhuri *et al.* 2007). Based on the ability of zAID to catalyse CSR in mouse cells, the model that AID activity might be modulated *in vivo* via a phosphorylation and RPA-dependent mechanism has been called into question by the Honjo and Goodman laboratories based on the observation that zAID lacks a PKA consensus site equivalent to S38 (Shinkura *et al.* 2007; Pham *et al.* 2008). On the other hand, we had noted that zAID has an aspartate residue at position 44 (D44) that might serve as a 'constitutive' mimetic of phosphorylation at S38 of mouse AID (Basu *et al.* 2005). Notably, all species known to undergo CSR and SHM (humans, mice, amphibians) have a conserved

Figure 2. Schematic representation of the modes by which AID activity is controlled. AID transcription is tightly controlled in B cells by various transcription factors and pathways. In addition, AID mRNA levels in B cells are negatively regulated by the micro RNA miR-155. Phosphorylation of AID at S38 and various other residues has been reported to control its DNA deamination activity. Nuclear levels of AID are regulated by ubiquitination followed by proteosomal degradation. The latter two examples describe the post-translational control of AID via modification(s). Although AID functions in the nucleus, most cellular AID is sequestered to the cytoplasm. AID harbours nuclear export and nuclear localization signals that have now been characterized.

PKA phosphorylation site centred at S38 (or its equivalent; figure 1). Strikingly, bony fish, which are not known to undergo CSR, lack a consensus PKA site centred on an S38 equivalent residue but instead have the conserved D44 residue or its equivalent (Barreto *et al.* 2005; Wakae *et al.* 2006; figure 1).

Recently, we have directly addressed the hypothesis that zAID D44 serves as a potential mimetic of phosphorylated S38 in mouse (Basu *et al.* 2008). In this regard, we have demonstrated that zAID constitutively associates with RPA in the absence of PKA phosphorylation and that association depends on integrity of zAID D44. Moreover, zAID is constitutively active in an *in vitro* transcription-dependent dsDNA deamination assay, as might be predicted by constitutive RPA association. Likewise, integrity of zAID D44 was required for full zAID activity in a retroviral complementation assay for CSR in AID-deficient mouse B cells. Remarkably, reconstitution of a PKA site into zAID in which the D44 residue was inactivated via an alanine mutation, generated a mutant AID that became phosphorylatable by PKA, showed PKA-dependent RPA-association plus PKA-dependent activity in the transcription-dependent dsDNA deamination assay and which had *in vivo* CSR activity that approached that of the WT zAID. The proposed functions of RPA in modulating AID activity have been difficult to test *in vivo* given the critical role of RPA in basic cellular processes such as DNA replication. However, our ability to link an AID second-site mutation to simultaneous gain of PKA phosphorylation-dependent RPA binding, PKA-dependent *in vitro* dsDNA deamination activity and augmentation of *in vivo* CSR activity strongly supports the linkage of these three phenomena. Finally, we have used the information from zAID to generate mouse AID mutants that function as a mimetic of AID phosphorylated at S38 (Basu *et al.* 2008).

8. SIGNIFICANCE AND FUTURE DIRECTIONS

Modest increases in AID levels can significantly affect CSR (Ramiro *et al.* 2006) and, moreover, can have

even greater effect on AID mutator activities, such as promoting translocations (Dorsett *et al.* 2008; Teng *et al.* 2008), potentially leading to evolutionary fixation of post-translational control mechanisms to more tightly regulate AID activity (Dorsett *et al.* 2008; Teng *et al.* 2008). The regulation of AID via phosphorylation at S38 may add to a growing list of mechanisms that regulate AID that include its transcriptional control, post-transcriptional control by miRNA155 (Dorsett *et al.* 2008; Teng *et al.* 2008) and its regulation via nuclear transport and proteosomal degradation (McBride *et al.* 2004; Aoufouchi *et al.* 2008; figure 2). In this context, it is not clear whether AID regulation via S38 phosphorylation in higher vertebrates that undergo CSR evolved from a 'constitutively' active AID, via the D44 equivalent in bony fish that do not undergo CSR, or if these two forms of AID represent different evolutionary paths from a common ancestral AID species. However, the notion that regulation of AID activity via S38 phosphorylation evolved in concert with CSR, a DSB-generating and therefore dangerous process, is intriguing.

It is notable that the zAID^{D44A} mutant, which does not bind RPA, catalyses significant CSR (approx. 50% of WT levels) when expressed in mouse B cells and can catalyse substantial gene conversion of variable region exons in chicken DT40 cells (Chatterji *et al.* 2007). While the residual *in vivo* CSR activity of zAID^{D44A} and mAID^{S38A} may reflect RPA-independent access to ssDNA in S regions in the context of R-loops (Longerich *et al.* 2006; Basu *et al.* 2007; Chaudhuri *et al.* 2007), access to variable region exons, and potentially S regions as well, may also employ additional, potentially related mechanisms. In this context, recent studies using a yeast model suggest that suboptimal co-transcriptional messenger ribonucleoprotein assembly in particular DNA regions may promote AID targeting by contributing to R-loop formation (Gomez-Gonzalez & Aguilera 2007) and similar mechanisms have been proposed for mammalian cells (Li & Manley 2006). An additional mechanism by which ssDNA may be generated at AID target substrates is through formation of stable stem-loop structures in negatively supercoiled DNA behind a translocating RNA polymerase II complex (Wright *et al.* 2004; Shen *et al.* 2005).

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