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Combinatorial Mechanisms Regulating AID-Dependent DNA deamination: Interacting Proteins and Post-translational Modifications

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Abstract

Protective humoral immune responses result from immunoglobulin (Ig) diversification reactions that proceed through programmed DNA double-strand breaks and mutations in developing or mature B cells. While primary Ig diversity is dependent on V(D)J recombination and the RAG proteins, secondary diversification is achieved through class switch recombination (CSR) and somatic hypermutation (SHM), which require AID (activation induced deaminase). Because aberrant AID activity can result in mutations in non-Ig loci and DNA translocations between the Ig locus and non-Ig genes, the activity of AID must be stringently regulated. AID mRNA expression is regulated transcriptionally by cytokine stimulation and post-transcriptionally by miRNAs. AID activity is regulated by post-translational modifications, subcellular localization, and interaction with other proteins. All of these molecular mechanisms have evolved to specifically induce AID-dependent mutations and DNA double-strand breaks at the Ig loci to promote maximal Ig gene diversification while limiting the access of this mutator to non-Ig regions.

1. INTRODUCTION

1.1. Overview

Mounting an effective humoral immune response requires the production of an almost infinite repertoire of immunoglobulins (Igs) that are capable of recognizing a diverse range of pathogens. In developing B cells, initial variable region diversity is generated through V(D)J recombination, whereby genes that encode the V, D, and J segments are shuffled into proximity by the RAG proteins (RAG1/RAG2) [1, 2]. Although V(D)J recombination generates a vast repertoire of Igs, this genomic reorganization fails to produce Igs with high affinity ($K_a 10^9 \text{ M}^{-1}$) toward their target antigens [3]. In addition, Igs produced solely through V(D)J recombination contain only a C μ constant coding region (i.e. IgM isotype) and therefore have limited effector functions (e.g. formation of pentameric polymers). In order to generate high affinity Igs with diverse effector functions, mature but naïve IgM-expressing B cells upon encountering antigens in secondary lymphoid organs undergo two additional programmed DNA alteration reactions – class switch recombination (CSR) and somatic hypermutation (SHM) [4-9].

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Occurring within the *IgH* locus, CSR is a DNA deletional-recombination reaction whereby the exons encoding the default C μ constant coding region are excised to juxtapose a different constant region gene (e.g. C γ , C ϵ , or C α) downstream of the rearranged variable region segment to produce IgG, IgE, or IgA [10]. SHM generates random mutations, and occasionally insertions or deletions, in the variable coding region of the recombined Ig heavy or light chain locus to alter the affinity of the immunoglobulin to its target antigen without affecting its effector function. Another related immunoglobulin diversification reaction, gene conversion (GC), which occurs in chickens, rabbits and sheep, generates templated alterations within the variable regions (reviewed elsewhere [11]). CSR, SHM, and GC are all dependent on AID (activation induced deaminase), a single-strand DNA cytidine deaminase, expressed primarily in activated B cells [12-15]. Cytidine deamination by AID generates G:U mismatches in DNA to initiate a series of reactions that ultimately lead to mutations in the variable region genes during SHM or breaks in the phosphodiester backbone of the DNA during CSR and GC [16-21].

1.2. Molecular Mechanism of CSR and SHM

CSR alters the isotype of the expressed *IgH* through a programmed DNA double-strand break (DSB) and end-joining reaction between two recombining switch (S) regions. S regions are 1-12 kb G:C rich, repetitive DNA sequences that precede the constant coding sequences (C H) for each Ig isotype [8-10]. The C H genes are organized as cytokine-inducible germline transcriptional units comprising of promoters, I H exons, S H regions, and C H exons [22, 23]. B cells activated for CSR induce germline transcription through S μ and a downstream switch region, such as S ϵ , that is poised to undergo recombination. Transcription through these S regions generates ssDNA in R-loop structures [24-27], which are targets of AID-mediated DNA deamination [17]. The deaminated cytosines are removed by UNG (uracil DNA glycosylase), a component of the base-excision repair (BER) machinery, to create abasic sites and subsequently APE-1 (apurinic/apyrimidinic endonuclease) nicks the DNA [16, 28-30]. In an alternative pathway, AID-induced G:U mismatches can be processed by the mismatch repair (MMR) machinery to generate nicks and gaps in the DNA [6, 16, 23] (see also Saribasak and Gearhart, and Chahwan et al. *this issue*). While the ssDNA of the non-template strand within R-loops is an optimal target of AID activity [17, 20], template strand deamination has been shown to be dependent in part upon the activity of the RNA exosome complex [31]. Overall, AID-mediated DNA deamination at both DNA strands and processing of the deaminated residues lead to the generation of staggered DSBs. Both the non-homologous end-joining (NHEJ) and a poorly characterized alternative-NHEJ pathways ligate DSBs between two distinct S regions to complete CSR [32-37].

Mechanistically, CSR and SHM are linked through the requirement of both transcription and AID [13, 14, 38-48]. However, V regions, unlike S regions, do not contain the G:C-rich sequences that stabilize ssDNA in the form of R-loop structures. In this context, AID interacts with the ssDNA binding protein RPA (Replication Protein A) and the AID•RPA complex, *in vitro*, binds to and deaminates transcribed DNA, such as V genes, that normally would not form stable R-loop structures [49]. These *in vitro* studies led to the proposal that during SHM of variable region genes, RPA binds to and stabilizes ssDNA within transcription bubbles, allowing AID-mediated deamination [49]. Consistent with this notion, mutations in AID that abrogate interaction with RPA (see section below on AID phosphorylation) significantly impair SHM without altering AID deaminase activity [50, 51].

The G:U mismatch created following AID deamination of V gene sequences can be processed in several ways to generate the observed SHM pattern [16]. Replication across the G:U mismatch generates transition mutations at G:C base pairs, while replication across a

UNG-created abasic site by error-prone DNA polymerases can lead to both transitions and transversions [16]. The G:U mismatch can also be processed by the MMR pathway, which with its associated error-prone DNA polymerases can induce mutations at both G:C and A:T base pairs [7]. Thus, upon inactivation of UNG, transversion mutations at G:C bases are dramatically reduced, while mutations in MMR genes (e.g., *Msh2*) lead to a significant reduction in A:T mutations [28, 29, 52]. In cells lacking both UNG and Msh2 activities, almost all mutations are transitions at G:C bases occurring via replication across the G:U mismatch [53]. The altered spectrum of SHM and severe defect in CSR observed in UNG and MMR mutant B cells [28, 29, 52-58] provide compelling evidence that AID-generated uridines in DNA serve as obligate intermediates of CSR and SHM.

1.3. Functions of AID outside of immunoglobulin diversification

Although much of the published work on AID has examined its role in Ig diversification, several recent reports suggest that the deaminase activity of AID, in concert with the BER pathway can mediate DNA demethylation during epigenetic reprogramming [59-61]. First, AID and the related DNA deaminase APOBEC1 were shown to deaminate cytosine and 5-methylcytosine (5mC) *in vitro* [62]. Second, in zebrafish, AID was shown to be in the same genetic pathway with the BER protein MBD4 and a scaffold protein GADD45a to promote DNA demethylation [63]. Third, in primordial germ cells, AID deficiency resulted in a significantly altered methylation profile [61]. Finally, using siRNAs in heterokaryons, AID was shown to be involved in promoter demethylation and induction of pluripotency associated genes *Oct4* and *Nanog* [59]. Taken together, these results provide compelling evidence that AID participates in active DNA demethylation and can partake in nuclear reprogramming towards pluripotency of somatic cells through deamination of 5mC, whereby the AID-induced T:G mismatch can be resolved by DNA glycosylases such as TDG (thymidine DNA glycosylase).

More recently it was shown that over-expression of AID can enhance demethylation of 5-hydroxymethylcytosine (5hmC), which is generated through oxidation of 5mC by the TET proteins [64], to initiate a process of BER that indirectly results in the demethylation of 5mC [65]. Although these remarkable studies have described a role of AID in active or passive demethylation, the physiological significance of AID-induced DNA demethylation of 5mC or 5hmC remains to be determined, as AID-deficient mice develop without any gross abnormalities of major organs [14]. Interestingly, AID-deficient female mice crossed to AID-deficient males produce smaller litters as compared to their wild-type counterparts; however, whether heritable epimutations are responsible for this phenotype remains to be determined [61]. In addition, the molecular mechanism by which AID accesses hypermethylated DNA to deaminate 5mC or 5hmC within the context of ssDNA remains unclear. Nevertheless, these and other reports have sparked extensive scientific inquiry into the function of AID beyond immunoglobulin gene diversification.

2. REGULATION OF AID EXPRESSION AND LOCALIZATION

While Ig loci are the primary targets of AID, several other genes are also mutated by AID [66-75]. These non-Ig targets of AID are of particular relevance to B cell lymphomagenesis, as a number of mature B cell lymphomas harbor mutations and translocations of oncogenes, such as c-Myc and Bcl6, that have been directly linked to AID activity [67, 69-73]. Thus, multiple mechanisms exist to restrict the expression and deamination activity of AID in B cells undergoing CSR or SHM.

2.1. Transcriptional regulation of AID

The discovery of AID through subtractive hybridization as a molecule upregulated in cells undergoing CSR suggested that its expression is modulated [12]. Encoded from the *Aicda* locus, AID is expressed primarily in activated germinal center B cells [12, 76], even though low levels of AID have been detected in early developing B cells in the bone marrow [77], germ cells, embryonic stem cells, and breast and ovarian tissue [59, 62, 78]. Regulation of *Aicda* expression has most extensively been studied in mature B cells. Binding sites for multiple transcription factors were identified both at the promoter and at regions upstream and downstream of the transcription start site, suggesting that a balance of activators and repressors regulate *Aicda* transcription in B cells [78-82] (see Orthwein and Di Noia *this issue*). The *Aicda* promoter just upstream of the transcription start site has binding sites for generally expressed activators, such as Sp1, NF κ B and HoxC4, and putative binding sites for the estrogen and progesterone receptors [78-86]. In naïve, unactivated B cells, repressor proteins E2f and c-Myb bind to a “silencer” region located within intron 1 of the *Aicda* locus. E2A proteins and Pax-5 can bind to this intronic sequence to partially alleviate the repression but cannot induce *Aicda* expression to the extent observed in activated B cells upon cytokine stimulation [82]. The cytokine-induced enhancer upstream of the transcriptional start site is bound by NF- κ B, SMAD 3/4, STAT6 and C/EBP to counter the repressive activity of intron 1 to amplify *Aicda* transcription. Thus, the ubiquitous expression of the repressor proteins and B-cell specific expression of the activators ensures that *Aicda* expression is induced only in cytokine stimulated B cells (reviewed in [87]).

Estrogen and progesterone have been shown to have opposing regulatory effects on *Aicda* transcription. B cells stimulated with estrogen upregulate *Aicda* transcription, which enhances CSR and SHM *ex vivo* and *in vivo*, whereas progesterone treatment represses *Aicda* transcription, CSR, and SHM [78, 79, 81]. A progesterone binding element (PRE) was characterized within the *Aicda* promoter that functions to repress TNF α -dependent *Aicda* transcriptional activation without displacing NF κ B activation complexes, which are assembled distal to the PRE [81]. Initial analysis of a 1.5kb promoter upstream of the *Aicda* transcription start site suggested that a putative estrogen response element (ERE) functioned independently and synergistically with the 5' proximal NF κ B sites to regulate *Aicda* transcription [78]. However, subsequent studies using mice deficient for *Hoxc4* demonstrated that estrogen-mediated transcriptional activation of *Aicda* in B cells requires HoxC4 binding to a conserved HoxC4/Oct-binding site within the 1.5kb promoter [79]. These data suggest that hormonal regulation of AID expression through estrogen and progesterone may function to improve humoral immune responses in females, which may help to protect them and their fetuses (or newborns) during their reproductive cycles, but also implicates dysregulated AID expression in the generation of autoantibodies, autoimmune disorders, or neoplastic transformation of non-B cells that are disproportionately found in females [88, 89].

2.2. Regulation of AID expression by miRNA

The 3'- untranslated region (3'UTR) of AID mRNA binds two miRNAs, miR-155 and miR-181b, and both these miRNAs have been shown to regulate the mRNA and protein expression of AID [90-92]. miR-155 and miR-181b were identified as miRNAs upregulated during CSR. Mutation of the miR-155 binding site in the 3'-UTR of AID mRNA, either in the context of a bacterial artificial chromosome (BAC) transgene or in the endogenous *Aicda* locus led to enhanced steady-state levels of AID, which increased CSR and *IgH-cMyc* translocations. Strikingly, there was no increase in SHM at the V region genes even though mutations at Bcl-6 were enhanced in the mutant B cells [90, 91]. Overexpression of miR-181b in primary B cells caused a 50% reduction in CSR to IgG1 with a corresponding decrease in AID mRNA and protein [92]. B cells from miR-155 deficient mice have a 50%

reduction in CSR despite normal levels of AID expression, suggesting that miR-155 regulates the expression of additional proteins required for CSR [91, 93]. As miR-181b levels decrease while miR-155 levels increase upon stimulation for CSR, the concerted actions of both miRNAs likely have nonoverlapping roles in regulating AID expression and consequently AID activity at non-Ig sequences, such as *Bcl6* and *Myc*.

2.3. Subcellular localization of AID

The majority of AID is localized to the cytoplasm due to Crm1/Exportin1-dependent active nuclear export of AID through the C-terminal nuclear export sequence (NES) [94-98] (also see Orthwein and Di Noia and Häslér, Rada, and Neuberger *this issue*). Remarkably, mutations that impair nuclear export also impair CSR without affecting SHM [96, 99, 100] and reduce AID protein stability, which may be a result of increased ubiquitylation of AID in the nucleus (see section on AID ubiquitylation) [97, 98, 100]. Although these data suggest that association of AID with Crm1/exportin1 and the nuclear export machinery is critical for the activation of CSR, AID binding to exportin1 is not a determinant for CSR, as AID chimeric proteins in which the NES of AID is replaced with an NES from another protein cannot reconstitute CSR in AID-deficient B cells [100, 101]. Hence, the relationship between the nuclear export of AID and efficient CSR remains an enigma.

2.4. AID Ubiquitylation and protein degradation

Ubiquitylated forms of AID are detected primarily in the nucleus of cells treated with the proteasome inhibitor MG132 [97]. Surprisingly, no specific lysine residue within the AID polypeptide sequence could be identified as the site of polyubiquitylation, as a mutant form of AID in which all eight lysines were mutated to arginine still had detectable ubiquitylation in the presence of MG132. AID was also found to be ubiquitinated in the cytoplasm upon pharmacological inhibition of its interaction with Hsp90 using geldanamycin [102]. More recently, REG- γ , a protein that regulates the levels of p21^{Cip1} independent of ubiquitin [103], was found to interact with nuclear localized AID to promote its degradation [104] (Figure 1). As compared to wild-type cells, REG- γ -deficient B cells displayed an increase in CSR, which is concomitant with an increase in AID protein levels [104]. Regardless of whether AID is degraded in a ubiquitin-dependent or -independent pathway, these reports clearly underscore the importance of regulating the levels of nuclear AID in order to maximize deamination, mutations, and DSBs at Ig loci while minimizing these events at non-Ig loci.

3. ASSOCIATION OF AID WITH DNA

Since the discovery of AID and its characterization as a general mutator, significant effort has been focused on elucidating the mechanism that specifically promotes the binding of AID to the Ig locus during CSR and SHM (Figure 2). In B cells, AID exists in a high-molecular weight complex [17] and several interactors have been identified. The ssDNA binding protein, RPA (Replication Protein A), was shown to interact with AID and promote binding and deamination of AID to transcribed variable region genes *in vitro* [49]. As RPA can bind to single-stranded DNA in transcription bubbles [105], it was proposed that the AID•RPA complex binds to and stabilizes ssDNA of transcribed variable region genes, allowing AID-mediated deamination during SHM [49]. While binding of RPA to variable region genes in germinal center cells undergoing SHM has not been directly demonstrated, a point mutation in AID that abrogates interaction with RPA (see below) significantly impairs SHM [50, 106], supporting the notion that the AID•RPA interaction is critical for SHM. In addition to RPA, other proteins have been shown to regulate AID deamination of V genes during SHM. The germinal center-associated nuclear protein (GANP) is induced in germinal center B cells during an immune response and has been reported to recruit AID to V genes.

GANP-deficient B cells undergo normal CSR but are significantly impaired for SHM [107]. GANP binds V region transcripts, and this purportedly enhances AID binding to V region DNA [108].

A screen for proteins that specifically bind to 5'-AGCT-3' repeat sequences, which are prevalent in S regions, identified 14-3-3 proteins as factors that could recruit AID to S region DNA [109]. Several isoforms of 14-3-3 are upregulated in B cells undergoing CSR. B cells deficient in 14-3-3 γ or expressing a dominant negative form of 14-3-3 σ are impaired for CSR with an associated reduction in the amount of AID and PKA (protein kinase A), which phosphorylates AID at serine38 (see section 4), bound to activated S regions [109]. These data support a model whereby 14-3-3 proteins function to nucleate AID and its associated proteins at DNA sequences that are targeted for recombination.

The observations that CSR, SHM and mutations of non-Ig genes are tightly linked to transcription of the recombined or mutated DNA sequence [38, 43, 47, 110-113] and that AID interacts with RNA polymerase II (Pol II) [114] suggested that Pol II might facilitate the binding of AID to target DNA sequences. Consistent with this notion, an shRNA screen for effectors of CSR revealed that the Pol II-associated factor Spt5, which binds to stalled Pol II, is required for CSR [115]. Spt5 interacts with AID and depletion of Spt5 markedly reduces recruitment of AID to Ig and non-Ig sequences. ChIP-sequencing analyses showed that Spt5 co-localizes with stalled Pol II and AID and remarkably, occupancy of Spt5 on stalled Pol II sites is predictive of AID-dependent mutations at the corresponding DNA sequence [115]. Spt5 and Pol II are also found throughout the C μ coding region [115], suggesting that additional mechanisms exist either to dissociate AID from the Spt5-Pol II complex or to limit the ability of AID to deaminate the DNA at C μ (e.g. lack of R-loops). Interestingly, in *S. cerevisiae* Spt5 coimmunoprecipitates with proteins of the histone chaperone complex FACT [116], which was shown to regulate histone3 lysine4 trimethylation (H3K4me3) and DSB formation at S regions during CSR [117]. These observations demonstrate a critical role for Spt5 and its associated proteins (Pol II, FACT) in regulating AID targeting to specific DNA sequences, and the subsequent generation of DSBs or mutations at these sites, during CSR or SHM [115].

PTBP2 is an AID interacting protein that plays a pivotal role in CSR [118]. PTBP2, and its closely related family member PTBP1, are RNA-binding proteins that participate in alternative pre-mRNA splicing [119]. Expression of PTBP2 was initially thought to be restricted to the brain and is therefore also known as brPTB (brain PTB) and nPTB (neuronal PTB). Subsequently, the protein was detected in testis, liver, heart, lung, skeletal muscle, thymus [120], and recently in B cells [118]. Knock-down of PTBP2 in B cells significantly impairs CSR [118]. The defect in CSR is associated with a reduction in the amount of AID bound to S regions, thereby identifying PTBP2 as a novel AID interactor that influences CSR by recruiting AID to DNA [118].

The mechanism by which PTBP2 targets AID to the Ig locus during CSR is still unknown. Although CSR has been closely linked to both germline transcription and splicing of germline transcripts [121], it is unclear whether components of the splicing machinery or the spliced transcripts themselves (or both) are required for CSR. Current models of CSR posit that transcription generates ssDNA substrates for AID in the context of R-loops [6]. This R-loop dependent model for CSR requires that the primary, unprocessed germline transcript stably hybridizes to the template strand. If splicing occurs co-transcriptionally and efficiently, R-loop formation would be transient. PTBP2 could be recruited to S region DNA, maybe through interactions with other chromatin-associated factors to inhibit splicing, thus enhancing the stability of R-loops and facilitating CSR. While unaltered steady-state levels of spliced germline transcripts in PTBP2 knock-down cells [118] argue

against such a role of PTBP2, transient alterations in the half-life of R-loop DNA may not be detectable. Alternatively, the role of PTBP2 in CSR could be independent of its role in splicing. *In vitro* studies show that PTBP2 binds to sense and antisense S region RNA suggesting that PTBP2 may recruit AID to S regions through its interaction with the S region transcripts [118]. Interestingly, AID has also been shown to interact with CTNNBL1, a poorly characterized protein that has been purified in 14S splicing complexes [122]. Mutants of AID that cannot bind to CTNNBL1 show a significant reduction in CSR and SHM; however, DT40 and CH12F3 cells carrying gene-targeted deletions of CTNNBL1 display low-levels of SHM and normal CSR, respectively [122, 123]. Additional work is required to elucidate the precise function of PTBP2, CTNNBL1, and the splicing of germline transcripts in CSR and SHM.

4. PHOSPHORYLATION OF AID

AID can be phosphorylated at multiple residues—serine 3 (S3), threonine 27 (T27), S38, T140 and tyrosine-184 (Y184) [49, 51, 106, 124, 125] (Figure 3). The S38, T140 and Y184 sites were identified by mass spectrometric analyses of AID purified from activated splenic B cells [51, 124]. Below we summarize the requirement of phosphorylation at each of these residues and describe in depth the possible function of AID phosphorylation at S38.

4.1. Phosphorylation at S3

The S3 residue was identified as a site on recombinant AID that could be phosphorylated *in vitro* by protein kinase C (PKC) [125]. Mutation of S3 to alanine (AID^{S3A}) does not affect the DNA deaminase activity of AID [125]. Strikingly, expression of a non-phosphorylatable mutant (S3A or S3D) in AID-deficient B cells and fibroblasts increases CSR and SHM, respectively, suggesting that phosphorylation of S3 is an inactivating event [125]. Consistent with the enhanced activity of AID^{S3A} in CSR, B cells expressing the mutant protein displayed a significant increase in *cMyc/Igh* translocation frequency relative to B cells expressing wild-type AID protein [125]. Blocking the activity of the serine/threonine phosphatase PP2A with pharmacological inhibitors increases AID phosphorylation at S3 with a concomitant reduction in CSR [125]. Thus, phosphorylation of AID at S3 suppresses AID activity; however, the mechanism through which this phosphorylation regulates AID function has not yet been determined.

4.2. Phosphorylation at T140

Mutation of T140 to alanine in AID does not affect DNA deaminase activity [106]. B cells from mice expressing AID^{T140A} displayed a mild defect in CSR [106]. Interestingly, AID^{T140A} mice exhibited a more profound defect in SHM as the mutant B cells displayed a mutation frequency of approximately 40% of wild-type B cells [106]. The mechanism by which T140 phosphorylation modulates AID activity during SHM is not known, but the differential activities of AID^{T140A} in CSR and SHM is reminiscent of mutations in AID that affect SHM but not CSR (and *vice versa*) [99, 126]. Thus, phosphorylation of AID at T140 may modulate the ability of AID to interact with an unidentified factor(s) that is required for SHM but not CSR.

4.3. AID phosphorylation sites of unknown function

Whereas mutation of Y184 to alanine does not affect the ability of AID to mediate CSR, mutation of T27 impairs CSR [124, 127]. However, AID^{T27A} is significantly weaker as a ssDNA deaminase, suggesting that the catalytic site of AID is affected by the mutation [124]. Although the T27 residue can be phosphorylated *in vitro* with recombinant protein kinase A (PKA) [124, 127], there is no evidence that it is phosphorylated in activated B cells. Likewise, additional phosphorylation sites at S41 and S43 were detected in Sf9 cells

expressing AID but whether these residues are functionally relevant during CSR or SHM is not known [128].

4.4. Phosphorylation at S38

Phosphorylation of AID at S38 (AID-pS38) has been studied most extensively. Lying within a PKA consensus phosphorylation site, S38 can be phosphorylated *in vitro* and *in vivo* by PKA and *in vitro* by multiple isoforms of PKC (protein kinase C) [51, 106, 124, 127, 129]. AID-pS38 is indistinguishable from wild-type AID in its ability to deaminate ssDNA [124]. Remarkably, deamination of transcribed V genes *in vitro* is strongly dependent on phosphorylation at S38 [49, 124, 130]. Biochemical experiments demonstrated that AID-pS38 interacts with RPA [49, 124, 130]. The AID-pS38•RPA complex binds and deaminates V genes [49] and *Xenopus laevis* S regions [131], which are rich in RGYW motifs but do not form R-loop structures when they are transcribed *in vitro* by T7 RNA polymerase. These observations led to the suggestion that the AID-pS38•RPA complex, by virtue of the ssDNA binding ability of RPA, can stabilize ssDNA within transcription bubbles at V genes, thereby generating AID substrates during SHM [49]. In addition, because RGYW motifs, and in particular the AGCT sequence, are highly enriched in mammalian S regions, the RPA-dependent mechanism of generating AID substrates was suggested to be important for CSR [49].

Consistent with predictions derived from *in vitro* activities of pS38-AID, an S38A mutant of AID (AID^{S38A}) fails to interact with RPA and is severely compromised in its ability to reconstitute CSR when expressed via retroviral transduction into AID-deficient B cells [50, 51, 124, 127]. Unequivocal demonstration that the S38 phosphorylation site is critical for AID function came from gene targeting studies. B cells from mice with an S38A knock-in mutation (*Aicda*^{S38A}) are substantially impaired in mediating both CSR and SHM [50, 106]. The defect is particularly pronounced in mice haploinsufficient for *Aicda*^{S38A} where the frequency of CSR and rate of SHM are reduced to near background levels [50, 106].

Several observations suggest that it is the failure to phosphorylate AID at S38, rather than a requirement of the serine residue *per se* that leads to the CSR and SHM defect in *Aicda*^{S38A} mice. First, AID^{S38A} is indistinguishable from wild-type AID in its ability to deaminate ssDNA, indicating that the mutation does not impair the core catalytic activity of the protein [124, 129]. Second, unphosphorylated wild-type AID, or AID^{S38A} which cannot be phosphorylated by PKA *in vitro*, does not interact with RPA and cannot mediate deamination of transcribed, double-stranded V region DNA despite retaining full ssDNA deaminase activity [124]. Third, genetic manipulation of PKA subunits that lead to a reduction of PKA activity in B cells decreases levels of AID phosphorylated at S38 and impairs CSR [129]. Finally, second site mutations in AID^{S38A} that restore RPA binding in the absence of AID phosphorylation significantly rescue the CSR defect of AID^{S38A} protein [130]. With regards to this latter point, zebra fish AID (zAID), which can partially restore CSR activity in AID-deficient cells, lacks an amino acid equivalent to mammalian S38 [132, 133]; however, zAID binds RPA constitutively, due to an aspartatic acid residue (D44) that mimics the phosphorylated S38 [130]. Remarkably, a D44A mutant of zAID cannot bind RPA nor restore CSR in AID-deficient B cells; however, a double mutant of zAID (G42S, D44A), which has lost the D44-dependent RPA binding but has gained a serine within the PKA consensus sequence that is required for RPA binding to mouse AID, restores RPA binding to zAID only in the presence of PKA and reconstitutes CSR comparably to wild-type zAID [130]. These results strongly suggest that the major, if not exclusive, activity of AID-pS38 is to interact with RPA.

Variable region genes, unlike S regions, are not G:C rich and thus do not form R-loop structures upon transcription. As discussed earlier, the AID-pS38•RPA interaction was

revealed in a biochemical screen for factors that would allow AID access to ssDNA in transcribed variable region genes. Since S regions are also rich in AGCT sequences, which serve as SHM hot-spots, it was proposed that the AID-pS38•RPA complex could participate in the binding of AID to S region DNA during CSR [49]. Contrary to this prediction however, AID^{S38A}, which does not interact with RPA [50, 124, 129], was as competent as wild-type AID in binding S region DNA, suggesting that AID phosphorylation and RPA binding to AID is not required for the association of AID with S region DNA during CSR [129]. However, as predicted from *in vitro* studies [49], association of RPA with activated S regions is strictly dependent on AID-pS38, as B cells expressing AID^{S38A} fail to recruit RPA to recombining S regions [129, 134]. Taken together, these observations suggest that RPA participates in CSR but not at the level of AID recruitment to S region DNA.

Given the current data, one can only speculate on the possible roles of RPA in CSR. Bound to S regions, RPA may function downstream of deamination to recruit UNG or MMR proteins, which participate in converting deaminated cytidines to DSBs. RPA may also recruit proteins such as 53BP1 and H2AX to DSBs to promote synapsis between distal broken S regions prior to their ligation during CSR. The known requirements of UNG, mismatch repair proteins, 53BP1 and H2AX in CSR and the reported interactions of these proteins with RPA support a role for RPA downstream of DNA deamination [8]. The failure to recruit RPA to S regions could thus impair conversion of the deaminated residues into DSBs or the interaction between distal DSBs. Either or both defects could be manifested as the marked reduction in CSR observed in *Aicda*^{S38A} B cells [50, 106]. Ongoing CSR in *Aicda*^{S38A} mutants probably reflects the ability of unphosphorylated AID to bind and deaminate S regions, with a few of the deaminated residues being converted into nicks and DSBs to effect a productive recombination reaction with a downstream S region in an RPA-independent mechanism. Consistent with this notion, artificially generating DSBs in S regions allows low levels of CSR in the absence of AID [135].

4.5. Protein Kinase A (PKA)

As mentioned earlier, the S38 residue of AID constitutes a consensus PKA phosphorylation site and PKA can phosphorylate AID at this residue [124]. The PKA holoenzyme comprises of two catalytic and two inhibitory, regulatory subunits. Binding of cyclic AMP to the regulatory subunits releases and activates the kinase activity of the catalytic subunits. Chromatin immunoprecipitation (ChIP) experiments in CSR-stimulated B cells demonstrated that the PKA catalytic subunit (PKA-Cα) and regulatory subunit (PKA-R1α) are specifically recruited to S regions [129]. The recruitment of PKA-Cα and PKA-R1α to S regions is independent of AID, as the association is detectable in AID-deficient B cells. Additional ChIP experiments in B cells derived from mice genetically engineered to express a hypomorphic mutant of PKA-R1α, which reduces PKA activity, showed that efficient phosphorylation of AID at S regions is required for wild-type levels of RPA recruitment and CSR [129].

The mechanism by which the PKA subunits are ferried and tethered to the S regions is unclear. PKA may associate with an unknown protein (factor X, Figure 4) which could be a specific transcription factor, an adaptor protein such as 14-3-3 [109] or a completely uncharacterized molecule. Binding sites for the cAMP-response element binding protein (CREB), a well-characterized PKA substrate, have been found in the germline promoters of *S_μ* and *S_{γ1}* and CREB has been shown to regulate germline transcription downstream of transforming growth factor-β stimulation [136]. The recruitment of PKA to S regions may be mediated by AKAPs (A Kinase Anchoring Proteins) that could coordinate the enzymes and substrates required for CSR to permit efficient activation of AID by juxtaposing AID to its substrate (DNA), its activator (PKA), and its co-factor (RPA). Irrespective of the mechanism of PKA recruitment to S regions, the association of PKA with S regions is

consistent with the hypothesis that localized activation of PKA through cAMP microdomains direct PKA activity towards specific substrates. AID phosphorylation and activation by PKA directly at recombining S regions thus establishes a paradigm to direct the specificity of a generally expressed kinase for its substrate through the concerted recruitment of both to a specific subcellular compartment. Accumulating high concentrations of inactive AID on S regions prior to “synchronously” activating the protein by phosphorylation could thus provide the mechanism by which a high density of DSBs are created in a particular S region during CSR [129].

5. CONCLUDING REMARKS

Generating mutations across two different chromosomes during SHM and inducing DSBs across several kilobases of sequence on one chromosome during CSR, AID has the unique ability to promote DNA diversification unlike any other characterized protein involved in Ig diversification (e.g. RAG, TdT). Because AID has the power to radically alter the organization of DNA as well as the coding sequences of DNA, mechanisms must exist to limit its activity specifically at the Ig loci. Significant progress has been made in identifying transcriptional, post-transcriptional, and post-translational regulatory mechanisms. In addition, several factors that influence AID binding to DNA have been identified. However, a specificity-factor, if it exists, that dictates how AID is able to precisely induce very high levels of mutations and DSBs at Ig loci remains elusive. Identifying how AID gains access to non-Ig genes, the mechanisms that promote (or restrict) this access and the process through which normal error-free repair by the general DNA repair pathways are subverted to induce mutations and DSBs in the genome during CSR and SHM will constitute the next frontier of investigation on the biology of AID.

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HIGHLIGHTS

- AID is a ssDNA deaminase
- AID expression is regulated transcriptionally and post-transcriptionally
- Subcellular localization and post-translational modifications limit AID activity
- AID interacting proteins restrict AID activity primarily to Ig loci

Figure 1.

Mechanisms regulating AID protein levels and subcellular localization. Cytoplasmic AID is stabilized by Hsp90, whereas nuclear AID is degraded by ubiquitylation or interaction with REG- γ . AID enters the nucleus passively through nuclear pores or actively through its putative N-terminal NLS (nuclear localization signal). AID is actively exported from the nucleus through its C-terminal NES sequence, which binds to Exportin1.

Figure 2.
Proteins interacting with AID at S regions during CSR

Figure 3.

AID phosphorylation sites. The primary structure of AID is shown with the numbers below indicating the amino acid residues that demarcate the putative NLS, the cytidine deaminase catalytic domain, and the NES (nuclear export sequence). Sites of AID phosphorylation are indicated with vertical lines above the primary structure, where boxed amino acids indicate AID phosphorylation sites that were identified in B cells undergoing CSR.

Figure 4.

Model of AID activation and RPA recruitment during class switch recombination. Activation of CSR induces the recruitment of PKA and AID either independently or as a complex through a putative, unidentified targeting factor X. The PKA regulatory subunits (R) inhibit AID phosphorylation and keep AID inactive for CSR until a burst of cAMP induces the release of the catalytic subunits (C), which phosphorylate AID. Phosphorylated AID recruits RPA to S regions to activate the CSR cascade.