Binding of AID to DNA Does Not Correlate with Mutator Activity

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Binding of AID to DNA Does Not Correlate with Mutator Activity

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The DNA deaminase activation-induced cytidine deaminase (AID) initiates somatic hypermutation (SHM) and class switch recombination (CSR) by deaminating cytidines to uridines at V region (V) genes and switch (S) regions. The mechanism by which AID is recruited to V genes and S region DNA is poorly understood. In this study, we used the CH12 B lymphoma line to demonstrate that, although S regions can efficiently recruit AID and undergo mutations and deletions, AID neither binds to nor mutates the V gene, thus clearly demonstrating intramunomglobulin locus specificity. Depletion of the RNA-binding protein polypyrimidine tract binding protein-2, previously shown to promote recruitment of AID to S regions, enables stable association of AID with the V gene. Surprisingly, AID binding to the V gene does not induce SHM. These results unmask a striking lack of correlation between AID binding and its mutator activity, providing evidence for the presence of factors required downstream of AID binding to effect SHM. Furthermore, our findings suggest that S regions are preferred targets for AID and, aided by polypyrimidine tract binding protein-2, act as “sinks” to sequester AID activity from other genomic regions. The Journal of Immunology, 2014, 193: 252–257.

Abbreviations used in this article: AID, activation-induced cytidine deaminase; SHM, somatic hypermutation; CSR, class switch recombination; Ct, cycle threshold; DSB, double strand break; PKA, protein kinase A; PKA-Cα, catalytic subunit of PKA; pS38-AID, AID phosphorylated at serine residue 38; Ptbp2, polypyrimidine tract binding protein-2; RPA, replication protein A; S, switch; SHM, somatic hypermutation; siRNA, short hairpin RNA.

Materials and Methods

Cell culture and protein analysis

CH12 cells (21) were stimulated at a density of 0.25 × 10⁶ cells/ml for 96 h with anti-CD40 Ab (CTT) (1 μg/ml; HM40-3; ebBioscience, San Diego, CA), IL-4 (12.5 μg/ml; 404-ML; R&D Systems, Minneapolis, MN), and TGF-β1 (0.1 ng/ml; 240-B; R&D Systems). IgA⁺ cells were generated...
from CIT-stimulated CH12 cells by negative selection with anti-IgM MicroBeads (Miltenyi Biotec, San Diego, CA), followed by positive selection using anti-IgA biotin Ab (eBioscience) and Streptavidin MicroBeads (Miltenyi Biotec). The predominantly IgA+ cell population was subcloned by serial dilution.

**Flow cytometry and Western blotting**

Cells were stained with IgM–PE-Cy 7 (R6-60.2; BD Biosciences, San Jose, CA) and IgA-FTTC (C10-3; BD Biosciences) and acquired on an LSR II (BD Biosciences). Data were analyzed using FlowJo software (TreeStar, Ashland, OR). (Invitrogen, Grand Island, NY) was used for exclusion of dead cells. Whole-cell lysates were prepared in Nonidet P-40 lysis buffer (20 mM Tris [pH 7.5], 5% [v/v] glycerol, 150 mM NaCl, 5 mM 2-ME, and 0.5% [v/v] Nonidet P-40). The following primary Abs were used: anti-Ptp2 (ab57619; Abcam, Cambridge, MA), anti-AID (22), and anti-Gadph (loading control; 6C5; Millipore, Billerica, MA).

**Chromatin immunoprecipitation analysis**

Knockdown in CH12 cells was described previously (15). Chromatin immunoprecipitation (ChIP) assays were performed as described previously (15, 23). PCR amplification and detection were carried out on a Bio-Rad CFX96 system, and threshold cycle (Ct) values were calculated using CFX Manager software by setting the threshold within the linear phase using a log-amplification plot. For detection of specific DNA sequences in CH12 cells, the SYBR Green Supermix (Bio-Rad, Hercules, CA) was used for Sa and ps3 amplifications, whereas FastStart Universal Probe Mix was used for VDJ amplification along with the labeled Universal Probe #45 (both from Roche, Indianapolis, IN). Melt-curve analysis was used for SYBR reactions to verify the presence of a single amplicon of the correct size. ChIP “relative units” were calculated by first normalizing the Ct value of each Ab sample by the Ct value of the input sample (CtAb/Ctinput). The inverse of this normalized Ab Ct value was then taken (1/CtAb).

**Statistical analysis**

A two-tailed paired Student t test was used to assess the significance of ChIP data; p < 0.05 was considered significant. A z-test for comparing proportions of two independent groups was used to assess the significance of mutation frequencies; p < 0.05 was considered significant.

**Results**

**AID binds to S regions but not to V gene segments in CH12 cells**

Upon stimulation with CIT, the mouse B lymphoma line CH12 expresses AID and undergoes robust CSR to IgA (21, 24) (Fig. 1A, 1B). Given that CSR in CH12 cells is dependent on several of the known factors implicated in CSR of primary B lymphocytes (7, 15, 25), these cells serve as a bona fide model system to elucidate CSR. Despite the high frequency of CSR, CIT-stimulated CH12 cells do not undergo endogenous SHM (26).

To test whether the failure to undergo SHM is due to preferred recruitment of AID to the S regions as opposed to the V gene, we carried out ChIP experiments in CH12 cells with AID Ab, using histone H3 Ab as a positive control. We analyzed the DNA–protein complexes for the presence of the Sa, Igh V gene (VDJ), and Trp53 (which encodes p53; non-Ig control) genomic sequence using sequence-specific primers (Supplemental Fig. 1A, Supplemental Table II). AID did not associate with Igh regions in unstimulated cells or with p53 under any condition (Supplemental Fig. 1B, 1C, 1F). As expected, AID binding to Sa was readily observed in stimulated cells; however, no AID was detected at the Igh V gene segment (Fig. 1C, Supplemental Fig. 1F).

One plausible explanation for the observed specificities is that S regions, being rich in RGYW sequence motifs and having the ability to form ssDNA in the context of R-loops, are significantly better than V genes at recruiting AID (18, 19, 27, 28). Therefore, we hypothesized that in “terminally switched” IgA+ CH12 cells, which cannot undergo further CSR, the Igh V gene would have a better chance of competing favorably against the smaller recombinated S region for AID binding. To test this notion, we purified IgA+ cells from CIT-stimulated CH12 cells and subcloned an IgA+ clone by serial dilution (Fig. 1A). When grown in the absence of cytokines, the IgA+ clone expressed no detectable AID, whereas robust AID expression was induced upon stimulation (Fig. 1B). As in CH12 cells, AID did not associate with Igh regions in unstimulated IgA+ cells or with the Trp53 (p53; non-Ig control) genomic sequence under any condition (Supplemental Fig. 1B, 1C, 1F). However, contrary to our predictions, AID binding to the Igh V gene segment was not detected in CIT-stimulated IgA+ cells (Fig. 1C, Supplemental Fig. 1F). Instead, AID was found associated with the recombinated Sa–So DNA (Fig. 1C, Supplemental Fig. 1F). Thus, AID exhibits intra-Igh locus specificity with a bias for binding to S regions, not only in bulk CH12 cells undergoing CSR, but also in IgA+ cells in which the Igh V gene might be expected to have a better chance of competing favorably against the remaining S region for AID binding.

**Activated CH12 cells do not undergo SHM**

To test the possibility that the failure to detect AID at the V gene is not due to the transient nature of the interaction, we isolated genomic DNA from IgA+ cells and sequenced the expressed Igh V gene using sequence-specific primers (Supplemental Fig. 1A, Supplemental Table II). The rate of mutation of the Igh V gene in unstimulated IgA+ cells or in Ptbp2-depleted cells was similar to that observed for unstimulated IgA+ cells, suggesting that SHM was not induced (Fig. 2A, Supplemental Table 1A). In contrast, the recombinated Sa–So sequence in CIT-stimulated IgA+ cells had significant levels of mutations (Fig. 2B, Supplemental Table 1B). Furthermore, the original recombinated Sa–So junction present in the unstimulated IgA+ cell line underwent deletions to create novel S junctions (Fig. 2B, 2C). Thus, although AID in IgA+ cells bound to and mutated the remaining S region, it neither associated with nor induced SHM at the Igh V gene.

**Localization of AID at the Igh locus is altered upon Ptp2 depletion**

The DNA-binding protein Ptp2 interacts with AID and facilitates the recruitment of AID to S regions (15). We investigated the possibility that, in Ptp2-depleted cells, AID that fails to bind to S regions can be “retargeted” to the V gene segment. Using a short hairpin RNA (shRNA) directed against the 3′ untranslated region of Ptp2 mRNA, we knocked down Ptp2 expression in CH12 cells, with “scrambled” shRNA serving as a control (Fig. 3A). As expected (15), AID binding to S regions was significantly reduced (~2-fold, p = 0.003), and CSR was impaired (Fig. 3B, 3C, Supplemental Fig. 1G). Strikingly, the reduction in AID at S regions was accompanied by a significant increase (>6-fold, p = 0.01) in AID binding specifically to the Igh V gene of stimulated
Ptbp2-depleted cells (Fig. 3C, Supplemental Fig. 1D, 1G). AID was not substantially enriched at other regions of the \textit{Igh} locus, including 1 kb upstream and downstream of the rearranged VDJ (5\textsuperscript{\prime}Vh1-53 and 3\textsuperscript{\prime}Jh2, respectively), C\textsubscript{\mu}, and the \textit{I\mu} promoter (Fig. 3D). Surprisingly, AID was not detected at the expressed V\textsubscript{\lambda}L chain locus (Fig. 3D). This could be due to occlusion of the Ab-binding epitope while AID is bound to the L chain locus or, more interestingly, a suggestion that the level of Ptbp2 preferentially effects AID relocalization within the \textit{Igh} locus during SHM; thus, only the binding of AID to the S region and \textit{Igh} V gene exons is altered upon Ptbp2 depletion. Finally, AID was not found to be associated with control genomic sequences, such as \textit{Trp}53 (p53) or other non-Ig genes that were shown to be upregulated in B cells undergoing CSR (23) (Fig. 3E). Thus, when the association of AID with S regions is impaired, its interaction with the expressed \textit{Igh} V gene segment is specifically and significantly promoted.

**FIGURE 1.** AID expressed in IgA\textsuperscript{+} cells does not bind the expressed \textit{Igh} V gene. (A) Flow cytometry analysis of IgM (left panel) or IgA (right panel) expression on unstimulated (U) or CIT-stimulated (S) CH12 or IgA\textsuperscript{+} cells. (B) Western blot analysis of whole-cell extracts using AID and Gapdh (loading control) Abs. (C) ChIP analysis to detect binding of AID or histone H3 to \textit{Sp} and \textit{Igh} V gene (VDJ) in stimulated CH12 or IgA\textsuperscript{+} cells. Two independent experiments are shown. “ChIP (Relative Units)” is defined as the reciprocal of the quotient of the crossing threshold (Ct) value of specific Ab immunoprecipitation signal and the Ct value of input signal, with nonspecific IgG immunoprecipitation signal (background) subtracted from this value.

AID bound to the \textit{Igh} V gene in PTBP2-depleted cells is phosphorylated

AID is phosphorylated at serine 38 (S38), and mutation of S38 to alanine impairs the ability of AID to mediate SHM and CSR (23, 29–33). To determine whether AID bound to the V gene was phosphorylated at S38 (pS38-AID), we carried out ChIP experiments using pS38-AID–specific Ab (Ab) (23). In accordance with AID bound to \textit{Sp}, the amount of pS38-AID localized to \textit{Sp} was reduced in Ptbp2-depleted CH12 cells (Fig. 4A). This reduction was accompanied by a significant increase (~7-fold, \textit{p} = 0.05) in pS38-AID levels specifically associated with the \textit{Igh} V gene (Fig. 4A). Localization of pS38-AID to the \textit{Trp53} (p53; non-Ig control) genomic sequence was not amplified from pS38-AID samples (Supplemental Fig. 1E). Thus, AID is not only specifically targeted to the V gene segment in Ptbp2-depleted CH12 cells, it is phosphorylated at S38.

AID phosphorylated at S38 interacts with the ssDNA-binding protein replication protein A (RPA) (29, 30). To test whether RPA localization also is altered in Ptbp2-depleted cells, we carried out ChIP analyses using an Ab specific for the 32-kDa subunit of RPA. RPA levels at \textit{Sp} were significantly reduced (~2.5-fold, \textit{p} = 0.03) in Ptbp2-depleted CH12 cells (Fig. 4A). In contrast, there was a significant increase (~3-fold, \textit{p} = 0.01) in RPA localization specifically to the \textit{Igh} V gene segment (Fig. 4A, Supplemental Fig. 1E).

Phosphorylation of AID at S38 is mediated by protein kinase A (PKA) (23, 30, 31, 34), and it is believed that AID is phosphorylated by S region–bound PKA to activate the CSR cascade (23, 35). In keeping with AID-independent recruitment of PKA to S regions (23), the catalytic subunit of PKA (PKA-C\textsubscript{\alpha}) was detected at \textit{Sp} in CIT-stimulated Ptbp2-depleted cells (Fig. 4B). However, PKA-C\textsubscript{\alpha} was not detected at the \textit{Igh} V gene segment (Fig. 4B), even though phosphorylated AID was readily detectable (Fig. 4A). Thus, not all proteins known to bind S regions are re-targeted to the V gene in the absence of Ptbp2, thereby ruling out the possibility that the binding of AID to the \textit{Igh} V gene is due to general deregulation of protein–DNA associations in Ptbp2-depleted CH12 cells.

Our findings that PKA-C\textsubscript{\alpha} is associated with S regions, but not with the \textit{Igh} V gene, regardless of Ptbp2 expression, lends credence...
to the proposal (23) that AID phosphorylation has a different role in SHM than in CSR. It is generally believed that transcribed S regions form R-loops, allowing AID to access S regions independent of its phosphorylation status (36). Phosphorylation of AID at S38 is still required to promote formation of DSBs at S regions through interaction with APE1 (35), as well as for the repair of DSBs through recruitment of RPA to S regions (8, 23). In contrast, transcribed V genes do not readily reveal ssDNA in the context of R-loops, and it is likely that AID only binds V genes in the context of a pS38–AID–RPA complex, because of the ability of RPA to bind and stabilize ssDNA within transcription bubbles (29). Our findings that pS38-AID and RPA, but not PKA-Cα, are detected at the IgH V gene support the notion that AID binding to V genes requires prior phosphorylation. However, we cannot exclude the possibilities that PKA-Cα was not detected at the V gene segment as a result of the transient nature of the interaction or that other proteins bound to the V gene segment mask the Ab binding site of V gene–bound PKA-Cα. Additionally, we cannot rule out the possibility that AID bound to the V region exons is not phosphorylated by PKA but is instead modified by an unidentified kinase.

AID localization to the V gene does not induce SHM

To determine whether AID binding induced SHM, we sequenced the IgH V gene (VDJ). Surprisingly, the mutation frequency in Ptbp2-depleted cells stimulated for 96 h was similar to that in scrambled cells (Fig. 5A, Supplemental Table I C). Moreover, Ptbp2-depleted cells stimulated continuously for 3 wk did not accumulate additional mutations (Supplemental Table I C). Although the mutation frequency was higher than in unstimulated cells (Supplemental Table I C), it was still considerably lower than typical SHM (32, 33, 37). The absence of SHM activity in Ptbp2-depleted cells cannot be attributed to a deficiency in IgH V gene (VDJ) transcription, because transcription through this region was not markedly altered (<2-fold change) upon Ptbp2 depletion (Supplemental Fig. 2A). Most importantly, there was no correlation...
of detectable SHM in Ptbp2-depleted CH12 cells is not due to a deficiency in the amount of available hot-spot motifs in this region; rather, it is likely due to the absence in expression of required repair mediators or uncharacterized SHM-specific factors in CH12 cells (Fig. 6). In conclusion, although known mediators of SHM, namely AID and RPA, were abundant at the IgV gene, and the bound AID was phosphorylated at S38, SHM was not induced.

Discussion

Our results clearly demonstrate that S regions are preferred targets for AID binding. Even in cells that have undergone CSR to the last C\(_\mu\) gene, AID is efficiently recruited to the remaining S region in lieu of the transcribed IgV gene. This strong preference for S regions might represent a physiological mechanism for AID regulation, wherein S regions act as a “sink” to prevent AID from interacting with non-Ig genomic sequences. Being noncoding, S regions could sustain mutations and deletions without having any deleterious effect on cell viability. This is evident from the abundant mutations and deletions observed at Sp-S\(_\alpha\) junctions upon re-expression of AID in IgA\(^+\) cells. Thus, active recruitment of AID to S regions probably functions as a default pathway to sequester AID activity away from other genomic targets.

If AID expressed in a B cell is indeed actively recruited to the S regions, the question that follows is how AID is targeted to the V genes to initiate SHM. Our studies clearly demonstrate that reduction in Ptbp2 levels promotes binding of AID to IgV gene segments. Thus, in a B cell undergoing SHM, modulating Ptbp2 expression or perturbing the interaction between AID and Ptbp2 through posttranslational modifications of AID and/or Ptbp2 could increase the amount of non-S region–bound AID (Fig. 6). The relative expression of Ptbp2 in germinal center B cells undergoing SHM versus CSR will shed further light onto the differential role of this protein during an immune response.

The most striking finding is that, despite binding to the IgV gene, AID cannot induce SHM. Over the past few years, several genome-wide association analyses of AID reported that it has the potential to bind to other genomic regions (7, 11, 39). Our findings clearly enforce the notion that binding of AID is not synonymous with mutations. For SHM to take place, at least in the context of CIT-stimulated CH12 cells, additional steps need to occur beyond AID binding. It is feasible that CH12 cells lack a factor(s) required for SHM and/or that CIT induction is not sufficient to induce the expression of all required proteins. Another likely possibility is that AID targeted to the IgV gene in Ptbp2-depleted cells is indeed actively deaminating, but high-fidelity repair (6), as
opposed to error-prone repair required for SHM, “fixes” the lesions in a nonmutagenic fashion. Under physiological conditions, SHM is associated with germinal center structures found in secondary lymphoid organs, where the interaction of B cells with CD4+ T cells (40) could induce SHM-promoting factors or localization does not determine AID deamination activity or the as an AID “sink,” sequestering AID away from the rest of the genome. Our findings identify Ptpb2 as a crucial mediator between S region and Ig H V gene AID targeting and show that AID deamination activity or the induction of mutation. Additionally, we describe a cell line–based model system that will be invaluable in further elucidating factors/conditions required for SHM versus CSR.

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Disclosures
The authors have no financial conflicts of interest.

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