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Amino-Terminal Phosphorylation of Activation-Induced Cytidine Deaminase Suppresses c-*myc*/*IgH* Translocation▽§

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Activation-induced cytidine deaminase (AID) is a mutator enzyme that initiates class switch recombination and somatic hypermutation of immunoglobulin genes (*Ig*) in B lymphocytes. However, AID also produces off-target DNA damage, including mutations in oncogenes and double-stranded breaks that can serve as substrates for oncogenic chromosomal translocations. AID is strictly regulated by a number of mechanisms, including phosphorylation at serine 38 and threonine 140, which increase activity. Here we show that phosphorylation can also suppress AID activity *in vivo*. Serine 3 is a novel phospho-acceptor which, when mutated to alanine, leads to increased class switching and c-*myc*/IgH translocations without affecting AID levels or catalytic activity. Conversely, increasing AID phosphorylation specifically on serine 3 by interfering with serine/threonine protein phosphatase 2A (PP2A) leads to decreased class switching. We conclude that AID activity and its oncogenic potential can be downregulated by phosphorylation of serine 3 and that this process is controlled by PP2A.

Antibodies are initially assembled in the bone marrow by *RAG1/2* recombinase-mediated site-specific recombination of immunoglobulin (Ig) variable (V), diversity (D), and joining (J) gene segments (17, 23). During immune responses, mature B cells further diversify *Ig* genes through somatic hypermutation (SHM) and class switch recombination (CSR). SHM alters antibody affinity by introducing nucleotide changes in the antigen-binding variable region of antibodies. B cells producing antibodies with improved antigen affinity are positively selected during the process of affinity maturation (13). CSR is a region-specific recombination reaction that replaces one antibody-constant region with another, thereby altering antibody effector function while leaving the variable region and its antigen binding specificity intact (13, 40, 44, 53, 55). While CSR and SHM are very different reactions, both are initiated by activation-induced cytidine deaminase (AID) (33, 49), which introduces uracil · guanine mismatches in transcribed DNA (4, 8, 12, 42, 48).

AID initiates SHM and CSR by programmed DNA damage at Ig loci. However, AID can also induce "off-target" DNA damage, including point mutations in oncogenes such as *bcl6* and c-*myc* (27, 37, 52), as well as double-stranded breaks that result in oncogenic chromosome translocations such as those between c-*myc* and *IgH* (c-*myc*/*IgH*) (46, 47, 50). Thus, maintaining genomic integrity requires strict control of AID activity; this is achieved at several different levels (19, 28). For example, AID gene transcription is mainly restricted to activated B lymphocytes (34). Once transcribed, the stability and half-life of the mRNA is controlled by microRNA-155 and microRNA-181b (11, 14, 54). Following translation, AID activity is regulated by phosphorylation, ubiquitination, and nuclear transport (1, 5, 18, 29, 38).

To date, biochemical experiments have demonstrated that phosphorylation is a positive regulator of AID activity (3, 6, 30, 36, 43). Phosphorylation of serine 38 or threonine 140 increases AID activity, and mutation of either of these residues to alanine leads to a hypomorphic phenotype, despite normal levels of protein expression and catalytic activity (9, 31). Although the mechanism by which threonine 140 phosphorylation enhances AID activity is not known, serine 38 phosphorylation is thought to increase activity by facilitating AID's interaction with chromatin, possibly by binding to replication protein A (3, 30). Less is known about the regulation of AID phosphorylation or dephosphorylation (30, 45, 57). While both protein kinase C (PKC) and cyclic AMP (cAMP)-dependent protein kinase A (PKA) can phosphorylate AID, the *in vivo* signaling pathways that impact AID phosphorylation have not been determined and no phosphatase has been reported to influence AID phosphorylation (3, 31, 36).

Here we identify a novel mechanism of AID regulation by phosphorylation of serine 3, which, in contrast to serine 38 or threonine 140, acts to suppress AID activity. We show that phosphorylation of serine 3 is controlled by protein phosphatase 2 (PP2A).

MATERIALS AND METHODS

Protein analysis. Anti-AID antibodies were previously described (30, 31). To produce anti-pS3 antibodies, rabbits were immunized with phosphopeptide MD(pS)LLMKQC (AID 1 to 8) coupled to keyhole limpet hemocyanin. Phos-

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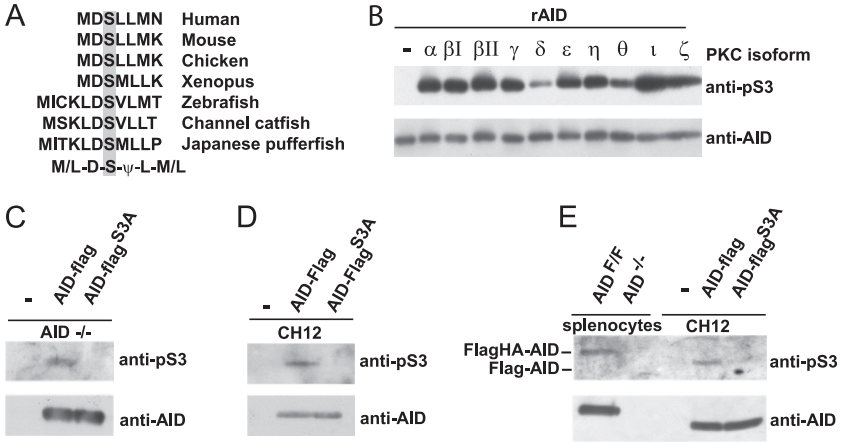

FIG. 1. AID is phosphorylated on serine 3. (A) Sequence alignment of the amino termini of human, mouse, chicken, frog, zebrafish, pufferfish, and catfish AID. The consensus sequence surrounding serine 3 (gray) in AID is shown below. (B) Anti-pS3 and anti-AID immunoblot of recombinant AID (rAID) purified from *E. coli* that was untreated (−) or treated with the indicated PKC isoform *in vitro*. (C to E) Anti-pS3 and anti-AID immunoblot of Flag-tagged AID or AID-S3A immunoprecipitated with anti-Flag antibodies from retrovirus-infected IL-4 and LPS-stimulated $AID^{-/-}$ splenocytes (C) or the CH12 B-cell line (D) or IL-4- and LPS-stimulated $AID^{-/-}$ and $AID^{Flag/Flag}$ (F/F) splenocytes (E). $AID^{Flag/Flag}$ expressed a tandem Flag-HA tag on AID.

pho-specific antibodies were purified by negative selection on unphosphorylated peptide coupled to Sulfolink gel (Thermo Fisher Scientific), followed by positive selection on the phosphopeptide. Cells were extracted in lysis buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 25 mM NaF, 0.1 mM vanadate, and 1 mM dithiothreitol [DTT]). For immunoprecipitation, 1 mg of extracts was incubated with anti-Flag agarose beads (Sigma-Aldrich) and AID was eluted with 0.5 μg/ml of Flag peptide (Sigma-Aldrich) in lysis buffer. Western blots were performed on immunoprecipitated protein or total cell extracts with the indicated anti-AID antibody; anti-green fluorescent protein (anti-GFP) (Santa Cruz) was used as a loading control, and anti-phosphoserine PKC substrate (Cell Signaling) was used to blot for phosphoserine. To phosphorylate AID *in vitro*, purified recombinant AID protein was incubated with PKC isoforms (Calbiochem) as previously described (31). For *in vitro* dephosphorylation, recombinant phosphorylated AID was incubated with 1 U of purified PP2A (Upstate) for 30 min at 30°C in 50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM DTT, 0.2 mg/ml bovine serum albumin (BSA). Mass spectrometry analysis of phosphorylation was performed on *in vitro* phosphorylated recombinant AID as previously described (30).

Lymphocyte isolation, culture, and retroviral infection. Lymphocyte isolation, cultures, carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, retrovirus infection with pMX-mK-AID, and CSR to IgG1 analysis were as described previously (30, 31). Retroviral AID-Flag contained a Flag tag fused in frame to the carboxy terminus of AID. B cells were purified from mouse spleens by depletion with anti-CD43 beads (Miltenyi Biotec) and cultured in 25 μg/ml lipopolysaccharide (LPS) (Sigma-Aldrich) with 5 ng/ml interleukin-4 (IL-4) (Sigma-Aldrich). Cells were stained with APC anti-mouse IgG1 (BD Biosciences). Cells were treated with the phosphatase inhibitors endothall, calyculin, and okadaic acid (Calbiochem). For the NTZ-3T3 assay, pMX-mK-AID vector with the GFP coding portion removed was used.

PCR, mutation analysis, and translocation assay. The NTZ-3T3 assay and GFP gene mutational analyses were performed as previously described 9 days after retrovirus infection (29, 60). The c-*myc*-IgH translocation assay was performed as described previously (25, 47). In brief, $AID^{-/-}$ LPS- and IL-4-stimulated B cells were harvested 72 h after infection with pMX-mK-AID or pMX-mK-AID-S3A (31). Ficoll gradient removal of dead cells was performed, and 96 separate PCRs on genomic DNA from 10^5 cells were performed with primers that amplify derivative 15 c-*myc*-IgH translocations. Amplified translocations were confirmed by Southern blots with probes internal to the primers used in the PCR assay (47). The experiment was performed twice independently, and the *P* value was calculated using a two-tailed Fisher's exact test.

Q-PCR analysis. RNA was extracted using Trizol (Invitrogen), cDNA prepared using Superscript II reverse transcriptase (Invitrogen) and quantitative PCR (Q-PCR) was performed using Brilliant SYBR green QPCR master mix (Stratagene) as per the manufacturer's protocol. Reactions were performed in triplicate and analyzed with an MX3000P Q-PCR machine (Stratagene). Reactions were normalized to GAPDH. Primers used were as follows: μGLT forward, 5′-TAGTAAGCGAGGCTCTAAAAAGCAT; reverse, 5′-AGAACAGTCCAG TGTAGGCAGTAGA; IgG1 GLT forward, 5′-TATGATGGAAAGAGGGTA GCATTCACC; reverse, 5′-CTCCTTCCCAATCTCCCGTG.

***In vitro* deamination assay.** The AID catalytic assay in *Escherichia coli* was performed exactly as described previously (48). For the UNG cleavage assay, a 50-base oligonucleotide (5′-GGAATTGAGTTGGTAGGGTAGCTAGGAGG TAAGTAGGGAAGATGGATGAT-3′) was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The single-stranded DNA (ssDNA) oligonucleotide was incubated with purified recombinant GST-AID or AID-S3A, deglycosylated with UDG (New England Biolabs), treated with 0.1 M NaOH, and subjected to electrophoresis on 15% PAGE–urea gels (8).

RESULTS

AID is phosphorylated on serine 3. In order to identify additional potential sites of AID phosphorylation, we subjected purified recombinant AID (rAID) to phosphorylation *in vitro* by protein kinase C (PKC) and ascertained sites of phosphorylation by mass spectrometry. Phosphorylation was detected at previously characterized serine 38 (S38) and threonine 140 (T140) and additionally at serine 3 (S3). AID-S3 and its surrounding residues are highly conserved through evolution (Fig. 1A).

To determine whether AID-S3 is also phosphorylated *in vivo*, we produced anti-AID phosphoserine 3 antibodies (anti-pS3) that recognized recombinant AID only after phosphorylation with different isoforms of PKC *in vitro* (Fig. 1B). Specificity of the antibody was confirmed by Western blotting against retrovirus-expressed AID or AID with S3 mutated to alanine (AID-S3A) purified from infected $AID^{-/-}$ splenocytes or CH12 cells (Fig. 1C and D). Anti-pS3 antibody reacted with AID but not with AID-S3A (Fig. 1C and D). In order to demonstrate that AID-S3 phosphorylation occurs *in vivo* under physiological conditions, we purified AID from splenocytes stimulated to undergo CSR with LPS and IL-4. Immunoprecipitation of AID with polyclonal anti-AID antibody resulted in a nonspecific anti-pS3 background. We therefore used activated B cells from mice that carry a Flag-tagged AID allele

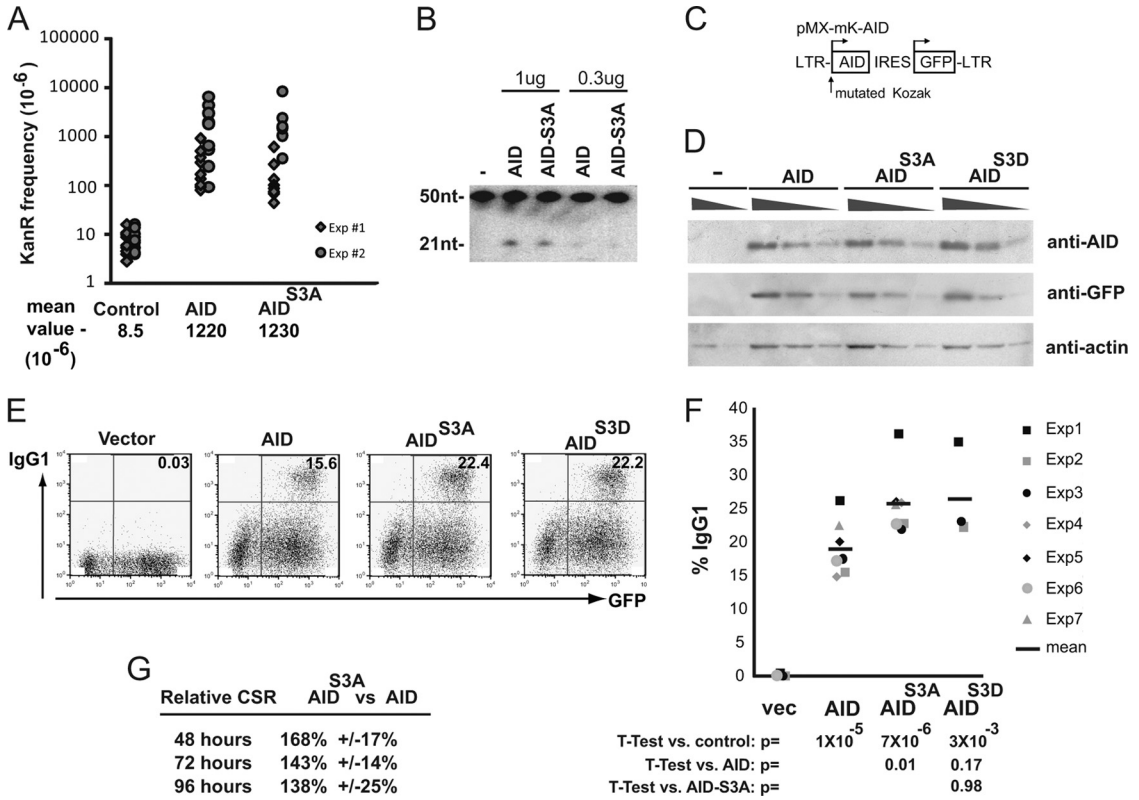

FIG. 2. Enhanced class switch recombination by AID-S3A. (A) Mutation measured by kanamycin resistance assay for AID-mediated cytidine deamination in UDG (uracil DNA glycosylase)-deficient *E. coli*. The graph shows a log plot of numbers of kanamycin-resistant (KanR) colonies after expression of AID or AID-S3A. Circles represent the mutation frequency of individual starting colonies. Results from two independent experiments are shown, and the mean frequencies of KanR colonies are indicated underneath each group. (B) *In vitro* deamination assay of single-target cytidine within ssDNA substrate. Denaturing PAGE gel of radiolabeled substrate incubated with recombinant purified GST-AID, AID-S3A, or a control reaction. The 50-oligonucleotide (nt) substrate and 21-nt product are marked. (C) Schematic representation of the retroviral construct used to express AID. (D) Anti-AID immunoblots of whole-cell extracts from LPS- and IL-4 stimulated AID$^{-/-}$ splenocytes transduced with pMX-mk-AID, -AIDS3A, or -AIDS3D. Three different dilutions are shown, and anti-GFP and antiactin were used as a loading control. (E) Representative fluorescence-activated cell sorter (FACS) plot of IgG1 expression of LPS- and IL-4-stimulated AID$^{-/-}$ splenocytes 72 h postinfection with pMX-mK-vector, -AID, -AIDS3A, or -AIDS3D. The relative percentage of GFP^{+} cells expressing IgG1 is in the upper right corner of each plot. (F) Flow cytometric analysis of IgG1 expression from seven independent experiments. Statistical significance was determined by a two-tailed *t* test assuming unequal variance. *P* values are indicated. (G) CSR to IgG1 by AID-S3A compared to CSR to IgG1 by AID at 48, 72, and 96 h postinfection. Results are for four experiments.

(AID$^{Flag/Flag}$) (39). *AID^{Flag}* is expressed at normal levels; the protein is active for CSR (39) and is phosphorylated on S3 (Fig. 1E). We conclude that AID is phosphorylated at S3 in activated B cells.

AID serine 3 phosphorylation in CSR. In order to determine whether S3 influences AID catalytic activity, we compared wild-type AID and AID-S3A for their ability to mutate a transcribed plasmid DNA in *E. coli*. This assay measures AID deamination by reversion of an inactivating point mutation in the kanamycin resistance gene (CCA^{P94} to CTA^{L94}), which results in kanamycin resistance (48). AID-S3A activity was indistinguishable from that of wild-type AID in this *E. coli* cytidine deamination assay (Fig. 2A). In addition, we compared in *vitro* enzymatic activity using a UNG-based deamination cleavage assay (7) (Fig. 2B). Consistent with the *E. coli* assay, purified recombinant AID-S3A and wild-type AID had similar deamination activities on an ssDNA substrate *in vitro* (Fig. 2B). Therefore, the mutation of S3 to A does not impact AID catalytic activity.

To examine the significance of AID-S3 phosphorylation *in vivo*, we compared class switch recombination in AID$^{-/-}$ B cells reconstituted with wild-type AID, AID-S3A, or AID-S3-to-aspartic-acid (AID-S3D)-expressing retrovirus. Since super-physiologic levels of AID expression lead to aberrantly enhanced class switching, we used a previously characterized retroviral construct with a mutated Kozak sequence (pMX-mKAID) that directs near-physiological levels of AID expression (31) (Fig. 2C). Wild-type AID, AID-S3A, and AID-S3D proteins were expressed at similar levels in the infected B cells as measured by Western blotting (Fig. 2D). However, AID$^{-/-}$ B cells expressing AID-S3A and AID-S3D showed a consistently higher percentage of class-switched IgG1-expressing B cells than control AID, irrespective of the time point assayed, ranging from 168 to 138% of the wild-type result (Fig. 2E through G). We conclude that mutating S3 to A does not alter catalysis but does result in increased AID activity in class switch recombination. The increased activity of AID-S3D indicates that phos-

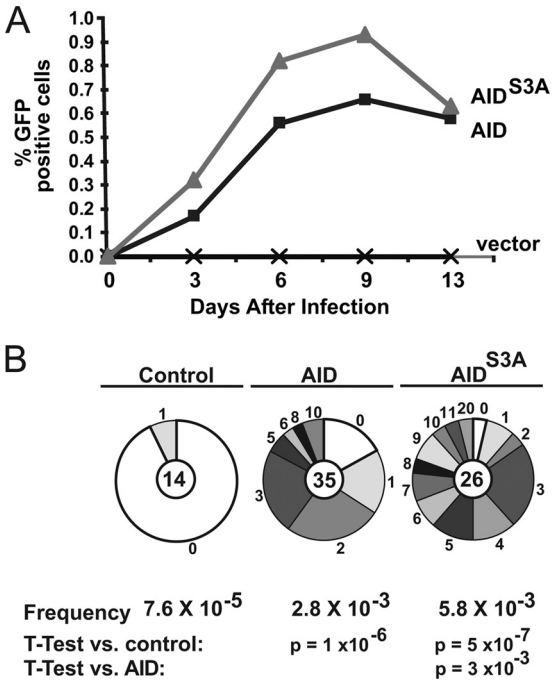

FIG. 3. Increased hypermutation in fibroblasts expressing AID-S3A. (A) GFP expression by 3T3-NTZ fibroblast cells after transduction with pMX-mK-AID or -AIDS3A expressing retrovirus. The *x* axis indicates the number of days after transduction, and the *y* axis indicates the percentage of GFP-positive cells measured by flow cytometry. (B) Number of mutations in the GFP gene cloned from 3T3-NTZ cells 9 days after transduction with retroviruses encoding AID, AID-S3A, or vector control. Segment sizes in the pie charts are proportional to the number of sequences carrying the number of mutations indicated in the periphery of the charts. The total number of independent sequences analyzed is indicated in the center of each chart. Statistical significance was determined by a two-tailed *t* test assuming unequal variance and comparing AID-expressing cells with AID-S3A-expressing cells. *P* values are indicated. Point mutations were as follows: 1 mutation in 13,174 bp mutations for vector, 92 mutations in 32,935 bp for AID, 142 mutations in 24,466 bp for AID^{S3A}.

phorylation contributes more than just a negative charge at this residue.

Hypermutation in fibroblasts. AID-induced somatic hypermutation can be measured in fibroblasts (3T3-NTZ) containing a green fluorescent protein (GFP) indicator with a premature stop codon which, when reverted by cytidine deamination, can be monitored by flow cytometry (60). Although wild-type AID and AID-S3A were expressed at similar levels, AID-S3A produced more GFP-positive cells than AID, suggesting a higher rate of hypermutation (Fig. 3A). To confirm the results found by flow cytometry and to determine whether AID-S3A displayed altered substrate specificity, we sequenced the green fluorescent protein gene from expressing 3T3-NTZ cells. AID-S3A showed an increased mutation frequency and number of clones with mutations compared to AID but no alteration in WRCY DNA hot spot motif recognition (Fig. 3B and data not shown). We conclude that mutation of AID-S3 to A results in increased hypermutation in the 3T3-NTZ fibroblast cell line.

c-*myc*/*IgH* translocation. In addition to *Ig* class switching, AID also produces double-strand DNA breaks that lead to oncogenic translocations between c-*myc* and *IgH* (15, 46, 47, 50). To determine whether AID S3 phosphorylation might also impact on the incidence of c-*myc*/*IgH* translocation, we assayed B cells expressing wild-type AID or AID-S3A for these aberrant chromosomes using a previously described PCR assay (47) (Fig. 4A). Consistent with its enhanced activity in class switching and somatic hypermutation, AID-S3A-expressing cells displayed a 2.5-fold increase in translocation frequency compared to the result for the wild type (*P* value = 1×10^{-7}; Fig. 4B and C). We conclude that cells expressing AID-S3A show higher levels of translocation than those expressing the wild-type proteins.

PP2A regulation of AID phosphorylation. To determine whether AID phosphorylation is regulated by a phosphatase, we treated the CH12 B-cell line with calyculin, a broad inhibitor with activity against both protein phosphatases 1 (PP1) and 2A (PP2A) and measured AID-S3, -S38, and -T140 phosphorylation by Western blotting. Whereas calyculin treatment did not alter S38 phosphorylation, it did result in increased AID-S3 and a more modest increase in AID-T140 phosphorylation (Fig. 5A). A similar increase in AID-S3 phosphorylation was observed in AID following calyculin treatment in stimulated splenocytes from $AID^{Flag/Flag}$ mice (Fig. 5B). In order to specifically examine the role of PP2A, we used okadaic acid (OA) and endothall, which are preferential inhibitors of this enzyme (10, 22, 26). We found that treatment of CH12 B cells with 300 nM okadaic acid or 50 μM endothall led to phosphatase inhibition as measured by Western blotting with anti-phosphoserine antibodies, as well as increased AID-S3 phosphorylation (Fig. 5C). To determine if PP2A could directly dephosphorylate AID-pS3 *in vitro*, we treated recombinant phosphorylated AID with purified PP2A. Anti-pS3 Western blot revealed a loss of AID-S3 phosphorylation (Fig. 5D). We conclude that PP2A is required for the regulation of AID-S3 phosphorylation.

To determine whether PP2A inhibition affected class switching, we treated activated primary B cells with okadaic acid or endothall for 24 h (Fig. 5E, F, and G). Treatment of 20 μM endothall or 10 nM okadaic acid did not impact cell viability, proliferation, IgM and IgG1 germ line transcription, or AID protein levels (see Fig. S1 in the supplemental material). However, both PP2A inhibitors reduced class switching to IgG1 (Fig. 5E and F) in both wild-type and $AID^{Flag/Flag}$ splenocytes while increasing AID phosphorylation at S3 (Fig. 5G; see Fig. S1 in the supplemental material). The phosphatase inhibitors also caused a similar decrease of CSR to IgA in stimulated CH12 cells (see Fig. S1). To determine if PP2A directly affects AID activity by impacting S3 phosphorylation, we measured CSR to IgG1 in $AID^{-/-}$ splenocytes expressing wild-type AID or AID-S3A following treatment with PP2A inhibitors (Fig. 5H). The relative percentage of CSR decrease compared to that of untreated cells was less in cells expressing AID-S3A than in wild-type cells (OA, $P = 0.05$; endothall, $P = 0.001$). These results are consistent with a role for PP2A in the regulation of AID-S3 phosphorylation and AID activity *in vivo*.

DISCUSSION

AID-induced programmed DNA damage is a double-edged sword: on the one hand, it produces high-affinity, isotype-switched antibodies; on the other hand, it also produces off-site

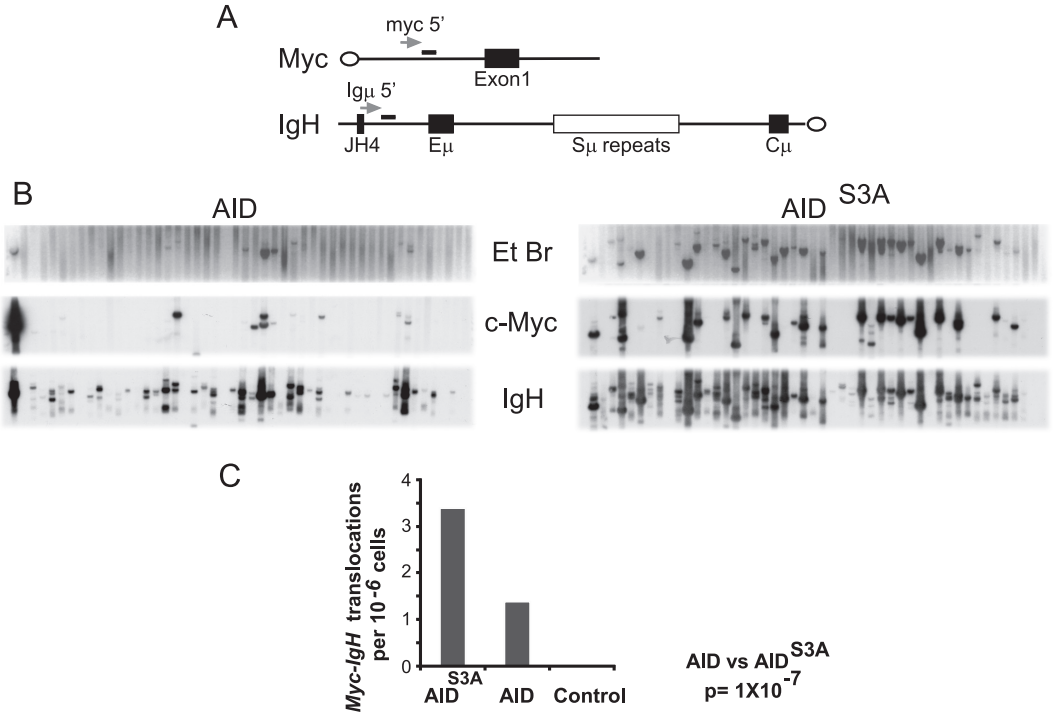

FIG. 4. c-*myc*/IgH translocations in AID- and AID-S3A expressing B cells. (A) Schematic representation of PCR assay for c-*myc*/*IgH* translocations. Primers used to detect translocations are represented as gray arrows. Internal probes used for the Southern blot analysis are shown as horizontal black bars, and centromeric location is shown as a circle. (B) c-*myc*/*IgH* translocations. Naïve B cells from AID$^{-/-}$ mice were stimulated with LPS and IL-4 and retrovirus transduced with pMX-mK-AID, -AIDS3A, or control vector (not shown). Translocations were assayed 72 h after retrovirus infection. Representative ethidium bromide-stained agarose gels and Southern blots with c-*myc* or *IgH* oligonucleotide probes are shown (10^5 cells per lane, 9.6×10^7 cells total, four spleens per retrovirus set from two independent experiments). (C) Frequency of translocations obtained from AID-S3A ($P = 1 \times 10^{-7}$ versus AID)-, AID-, and vector-control-infected B cells. The *P* value was determined with a two-tailed Fisher's exact test.

mutations and chromosome translocations (27, 35, 37, 46, 47, 50–52). AID can also be detrimental to antibody maturation, since persistent activity might lead to the loss of affinity or increased self-reactivity. Therefore, strict regulation of AID is necessary for the maintenance of genome integrity and proper antibody function. Genomic damage by AID is limited by its specific transcription, regulation of its mRNA levels by microRNAs (miRNAs), and restricted nuclear concentration through cytoplasmic retention, active nuclear export, and degradation (1, 5, 11, 14, 18, 29, 38, 54). In addition, AID is a phosphoprotein. Phosphorylation, in contrast to the aforementioned means of regulation, has the potential for rapid and dynamic modulation of AID activity.

In switching B cells, mass spectrometry analysis has detected phosphorylation on AID-S38, T140, and Y184 (3, 31). Additional sites at T27, S41, and S43 have been reported in Sf9 insect cells (43). The best-characterized sites, S38 and T140, are positive regulators of AID function. While the *in vivo* signaling pathways that impact AID phosphorylation are unknown, S38 is thought to be a PKA target. S38 is part of a PKA consensus site and can be *in vitro* phosphorylated by PKA, and coimmunoprecipitation revealed an association with PKA (3, 31, 36), which can be specifically recruited to the switch regions (57). Less is known regarding T140 phosphorylation, which is a target of PKC but not PKA (31). These results, together with the fact that PP2A preferentially influences S3 phosphorylation, suggest that AID activity is impacted by multiple signaling pathways.

In this study, prevention of phosphorylation by mutation of AID-S3 to AID-S3A results in a 40 to 70% increase in CSR (Fig. 2) and an ~3-fold increase in translocations (Fig. 2 and 4). In 3T3-NTZ fibroblasts, the AID-S3A results in a greater-than-2-fold increase in the rate of mutation (Fig. 3). Although hypermutation in fibroblasts is not equivalent to SHM in germinal center B cells, the results suggest that AID-S3 phosphorylation alters activity on transcribed DNA. The magnitude of the S3-to-A effect is similar in scale to the mutation of AID-S38 to AID-S38A, to the mutation of AID-T140 to AID-T140A, or to AID regulation by mir-155, each of which has significant effects on CSR, SHM, and translocation (9, 14, 30, 31, 54). Since only a small percentage of AID-S3 is phosphorylated in splenocytes stimulated to undergo CSR (Fig. 5A and B), our measurement of the effects of AID-S3 phosphorylation on AID activity may be an underestimation. Using PP2A inhibitors, we observed that increasing AID-S3 phosphorylation 3- to 4-fold (Fig. 5G) correlates with an ~60% decrease in CSR (Fig. 5E). Potentially even higher levels of AID-S3 phosphorylation could significantly or even completely abrogate AID activity. Thus, AID-S3 phosphorylation represents the first dynamic mechanism that could rapidly and significantly inhibit AID mutator function. We speculate that rapid inhibition of AID activity could be important in the germinal center

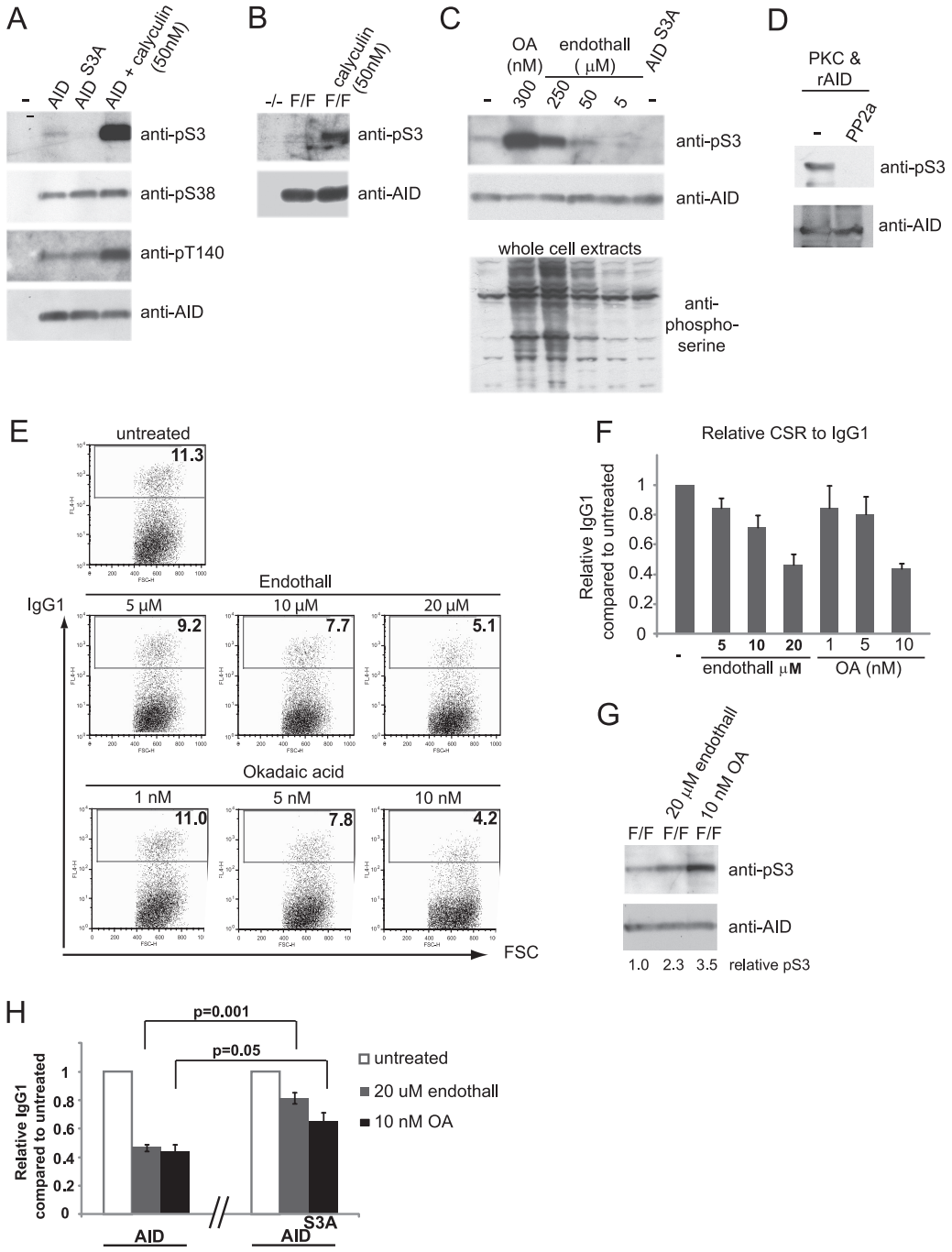

FIG. 5. PP2A inhibitors affect AID phosphorylation and CSR. (A) Anti-pS3, -pS38, -pT140, and -AID immunoblot of Flag-tagged AID or AID-S3A immunoprecipitated with anti-Flag from CH12 cells untreated or treated with 50 nM calyculin for 30 min. (B) Anti-pS3 and anti-AID immunoblot of Flag-tagged AID immunoprecipitated with anti-Flag from 72-h IL-4- and LPS-stimulated $AID^{-/-}$ or $AID^{Flag/Flag}$ (F/F) splenocytes alone or treated with 50 nM calyculin for 30 min. (C) Anti-pS3, and anti-AID immunoblot of Flag-tagged AID or AID-S3A immunoprecipitated from CH12 cells untreated or treated with 300 nM okadaic acid (OA) or 250 μM, 50 μM, or 5 μM endothall (top panel) for 1 h. An anti-phosphoserine blot of 50 μg of corresponding whole-cell extracts is shown in the lower panel. (D) Anti-pS3 and -AID immunoblot of PKC-phosphorylated recombinant AID (rAID) that was untreated (−) or treated with PP2A *in vitro*. (E) Representative flow cytometric plots of IgG1 expression by wild-type splenocytes stimulated for 72 h with LPS and IL-4. Cells were treated with the indicated concentration of inhibitors for 24 h before analysis. (F) Summary of effect of inhibitors shown in panel E from four independent experiments consisting of two wild-type and two (F/F) CSR analyses. The untreated sample was assigned an arbitrary value of 1.0. (G) Anti-pS3 and anti-AID immunoblot of Flag-tagged AID from 72 h IL-4- and LPS-stimulated F/F splenocytes alone or treated with 10 nM okadaic acid or 20 μM endothall for 24 h. Numbers indicate relative AID-S3 phosphorylation determined by AID-pS3/AID ratio measured by densitometry. (H) Effect of inhibitors on IgG1 expression in $AID^{-/-}$ splenocytes transduced with pMX-mK-AID or -AIDS3A, 72 h postinfection and 24 h after inhibitor treatment. Untreated AID and AID-S3A were both independently assigned an arbitrary value of 1.0, and treatment represents the relative decrease in IgG1 expression of treated compared to untreated cells. Results are summarized from three independent experiments, and statistical significance was determined by a two-tailed *t* test assuming unequal variance. *P* values are indicated.

to prevent accumulation of detrimental mutations following the expression of high-affinity, class-switched antibodies.

The mechanism by which phosphorylation of S3 interferes with AID function is not known; however, phosphorylation does not affect protein stability, as demonstrated by the fact that neither AID-S3-to-A mutation nor treatment with phosphatase inhibitors alters AID levels. In addition, AID-S3 mutation to AID-S3A does not alter AID activity in *E. coli* or *in vitro* on an ssDNA substrate; therefore, this phosphorylation is not likely to change catalytic activity directly (58). Instead, we observe increased AID-S3A levels at the switch μ region by chromatin immunoprecipitation (ChIP) analysis (see Fig S2 in the supplemental material), suggesting that phosphorylation interferes with *Ig* target substrate association. AID-S3 and the surrounding residues are highly conserved through evolution but do not correspond to a known kinase consensus site (Fig. 1A). PKC was used to uncover AID-S3 phosphorylation; however, it may not be the physiological kinase, as inhibitors of PKC and PKA do not completely inhibit phosphorylation (data not shown). However, PP2A appears to be a key regulator of the level of AID phosphorylation at S3, since defined pharmacological PP2A inhibitors augment AID-S3 phosphorylation *in vivo*. Calyculin inhibits both PP2A and PP1 equally (50% inhibitory concentration [IC_{50}], 1 nM), okadaic acid has a >100-fold-higher affinity for PP2A (IC_{50}, 1 nM) (10, 22), and endothall is a PP2A-specific inhibitor (26). Each of these inhibitors augments AID-S3 phosphorylation in stimulated B cells and also inhibits CSR (Fig. 5), which demonstrates a specific PP2A involvement in regulating these processes.

PP2A is a ubiquitous serine/threonine phosphatase that has broad specificity and diverse cellular functions. Specifically, PP2A is a heterotrimeric protein consisting of a common catalytic unit and structural units together with a regulatory unit (20). There are numerous regulatory units that are thought to impart specificity by controlling localization, activity, and interaction with substrates (56). These different regulatory subunits have differential expression levels and tissue expression patterns. Presently, it is unclear which of the PP2A regulatory subunits are responsible for regulating AID. Consistent with the notion that PP2A regulates the phosphorylation of numerous cellular proteins, its effects on CSR appear to be both direct and indirect. CSR was less affected in AID-S3A-expressing cells than in wild-type cells following endothall or OA treatment (Fig. 5H), indicating that PP2A does regulate AID function through S3. However, the fact that inhibitors decrease CSR in AID-S3A indicates that PP2A also regulates other proteins involved in CSR.

PP2A is an important regulator of signaling pathways involved in carcinogenesis, and its role as a tumor suppressor has been demonstrated in numerous studies. Inactivating mutations of PP2A subunits, overexpression of endogenous or viral PP2A suppressors, and treatment with inhibitors such as okadaic acid all promote cellular transformation (2, 16, 21, 32, 59). PP2A inactivation has also been linked to B-cell neoplasia in chronic myelogenous leukemia (CML), chronic lymphocytic leukemia, and acute lymphoblastic leukemia. Likewise, activators of PP2A, such as forskolin, antagonize lymphomagenesis (16, 32). For all these reasons, PP2A has been proposed as a target for therapeutic intervention (41).

Preventing AID-S3 phosphorylation leads to increased AID activity and translocations. We speculate that increased PP2A activity would have a similar effect. Regulation of AID by PP2A is of particular relevance to the treatment strategies of certain B-cell cancers involving PP2A inhibition. In CML, for example, AID expression promotes B lymphoid blast crisis, BCR-ABL1 mutation, and the acquisition of imatinib resistance (24). Therefore, therapy strategies centered on activating PP2A in these tumors may actually increase AID activity and genomic instability. In contrast, signaling pathways that impact AID-S3 phosphorylation may promote genomic integrity and thus may be potential targets for future studies of AID function in normal and abnormal B cells.

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