The immune system produces a diverse array of antibodies by a series of DNA transactions that require programmed DNA damage. Ig transcription units are assembled by random joining of variable (V), diversity (D), and joining (J) gene segments by a site-specific recombination reaction mediated by RAG1 and RAG2 proteins in developing B cells (1, 2). Later, during immune responses, antigen-specific clones of B cells are selectively expanded in structures called germinal centers (GCs), where they undergo Ig somatic mutation and class switch recombination (CSR) (3, 4). Somatic hypermutation (SHM) introduces nontemplate nucleotide substitutions into the Ig variable gene (5), which can alter the binding affinity of the antibody molecule. If the affinity is enhanced, the resulting B cell clone is selectively expanded, ultimately resulting in affinity maturation of the antibody response (3, 4).

CSR is a region-specific deletional recombination reaction that replaces one antibody-constant region for another, thereby altering antibody effector function (6–11). Class switching does not involve the variable regions, and therefore switched antibodies retain their antigenic specificities. Although somatic mutation and class switching are fundamentally different DNA transactions, they are initiated in the nucleus by the same enzyme, activation-induced cytidine deaminase (AID) (12–14), which introduces uracil:guanine mismatches in transcribed single-stranded (ss) Ig DNA (15–19).

In addition to its effects on Igss, AID produces off-target DNA damage, including mutations in oncogenes. Therefore, stringent regulation of AID is required for maintaining genomic stability during maturation of the antibody response. It has been proposed that AID phosphorylation at serine 38 (S38) regulates its activity, but this has not been tested in vivo. Using a combination of mass spectrometry and immunochemical approaches, we found that in addition to S38, AID is also phosphorylated at position threonine 140 (T140). Mutation of either S38 or T140 to alanine does not impact catalytic activity, but interferes with class switching and somatic hypermutation in vivo. This effect is particularly pronounced inhaploinsufficient mice where AID levels are limited. Although S38 is equally important for both processes, T140 phosphorylation preferentially affects somatic mutation, suggesting that posttranslational modification might contribute to the choice between hypermutation and class switching.
expression is restricted to activated B cells within GCs (13) by a requirement for PAX5 and E47 transcription factors (27, 28).

AID levels are limiting for CSR and hypermutation (29–31), and they are known to be regulated by microRNA-155, which controls the half-life of AID mRNA (30, 32). In addition, the concentration of AID in the nucleus is limited by a combination of active export and selective nuclear degradation (33–36). Finally, biochemical and tissue culture experiments indicate that a fraction of AID is posttranslationally modified by phosphorylation of serine 38 (S38) and that this modification may also regulate AID activity (29, 37, 38).

S38 is thought to be a target of c-AMP–dependent protein kinase A (PKA) because S38 is part of a PKA consensus site and can be phosphorylated in vitro by PKA (37). Furthermore, coimmunoprecipitation experiments showed that AID is physically associated with PKA (37, 38). In biochemical assays phosphorylation at S38 is essential for AID to associate with replication protein A (RPA), a single stranded DNA binding protein, and the interaction is required for AID to access actively transcribed DNA (37). Consistent with this observation, AID phosphorylated at S38 is enriched in chromatin (29).

Although the role of S38 phosphorylation has not been tested in vivo, mutating S38 to alanine (AIDS38A) results in catalytically intact AID, which may have an altered substrate preference in biochemical assays in vitro (29, 37, 39). Expression of AIDS38A has also been reported to variably decrease CSR, assayed in AID-deficient B cells in vitro (10–80% of wild type) (29, 37, 38, 40) and SHM and gene conversion in chicken DT40 B cells (15% of wild type) (41). Further confounding the question of the function of S38 phosphorylation is the fact that zebrafish AID lacks a serine at this position, yet retains activity in CSR and gene conversion (41–43).

In this study, we report on a novel site of AID phosphorylation at threonine 140 (T140) and examine the impact of S38 and T140 phosphorylation on CSR and SHM in vivo.

RESULTS

To examine posttranslational modification of AID, we purified the protein from B cells cultured with LPS and IL-4 and subjected the material to mass spectrometry. Analysis of purified AID confirmed phosphorylation at peptides containing S38 (p38), and tyrosine 184 (p184) (29, 37) and revealed additional phosphorylation at T140 (p140; Fig. 1 A).

To confirm that AID is phosphorylated at T140 in vivo, we produced AID-p140 phosphospecific antibodies (anti-p140). Anti-p140 was reactive with AID purified from B cells stimulated with LPS and IL-4, but was not reactive with AID-T140A (Fig. 1 B). The level of anti-p140 reactivity in cultured B cells differed depending on the stimulus with the highest levels found with CpG (Fig. 1 C). However, phosphorylation at AID-T140 was not B cell specific and could also be detected in NIH-3T3 NTZ cells expressing AID (Fig. 1 D). Finally, anti-p140 did not react with recombinant AID produced in bacteria, but was reactive with AID phosphorylated in vitro with protein kinase C (PKC) catalytic subunit and not PKA (Fig. 1 E). The PKA family is composed of at least 10 serine/threonine kinases, and anti-p140 and -p38 was reactive to varying degrees with recombinant AID after in vitro phosphorylation with the different isoforms (Fig. 1 F). We conclude that AID is phosphorylated at position T140 and that this site of posttranslational modification resembles S38 in that it is not B cell specific.

To determine whether T140 is essential for catalytic activity, we compared wild-type and AID-T140A for their ability to revert an inactivating point mutation in a kanamycin resistance encoding plasmid in Escherichia coli (19). Reversal of the point mutation (CCA to CTA) by cytidine deamination confers kanamycin resistance, which is assayed by colony formation (19). AID-T140A, AID-S38A, and the double mutant (AID-ST/AA) were indistinguishable from

Figure 1. AID is phosphorylated at position T140. (A) Amino acid sequence of AID showing the location of S38 and T140 within consensus PKA and PKC sites, respectively (gray boxes). (B) Anti-p140 or -AID immunoblot of recombinant AID (rAID) untreated (−) or treated with PKC or PKA in vitro. (C) Anti-p140 and -AID immunoblot of rAID untreated (−) or treated with PKC or PKA in vitro. (F) Anti-p140, -p38, and -AID immunoblot of rAID untreated (−) or in vitro phosphorylated with the indicated PKC isoforms.
wild type in this assay (Fig. 2 A) (29). Thus, these mutations do not alter AID catalytic activity as assayed in E. coli.

To determine whether there is interdependence between S38 and T140 phosphorylation, we expressed AID, AID-S38A, or AID-T140A in stimulated AID−/− B cells and performed Western blotting experiments with anti-p140 and -p38 antibodies. We found that AID-S38A was normally phosphorylated at position T140 and, conversely, that AID-T140A was normally phosphorylated at S38. We conclude that the two sites of phosphorylation are not interdependent (Fig. 2 B).

AID is phosphorylated in 3T3 fibroblasts (Fig. 1 D) and mutates indicator substrates when it is expressed in these cells. Mutator activity can be measured by reversion of a green fluorescent protein indicator containing a premature stop codon (NIH-3T3 NTZ cells [44]). When assayed by FACS or sequencing, AID-S38A and -T140A showed <15 and <50% of wild-type levels, respectively (Fig. 2, C and D) (29). We conclude that T140 is required for normal levels of mutation in NIH-3T3 NTZ cells in vitro.

AID can be assayed for its ability to induce CSR in B cells in vitro by retroviral complementation of AID-deficient B cells stimulated with LPS and IL-4 (45). Because AID expression levels may impact on the results of the assay, we used two retroviral vectors that directed different levels of AID expression (Fig. 2 E). B cell retroviral infection with PMX-AID (25) or MK-AID, which contains a mutant Koakz sequence, results in AID levels that are 10- or 2.5-fold higher than physiological levels, respectively (Fig. 2 E). Consistent with the high levels of AID expression, B cells infected with PMX-AID switched to IgG1 at higher efficiency than cells infected with MK-AID (Fig. 2, F and H). AID mutants showed varying degrees of activity in this assay (Fig. 2, F and H). High levels of AID-T140A expression reconstituted nearly 95% of control levels of class switching (Fig. 2, F and G). In contrast, lower levels of AID-T140A expression resulted in 50% of control levels of switching (Fig. 2, H and I). As previously reported, AID-S38A mutants varied in activity depending on expression levels ranging from nearly undetectable to 60% of control levels (Fig. 2, G and I) (29, 37, 38, 40). High-level expression of the double mutant, AID-ST/AA, resulted in a modest drop in class switching compared with AID-S38A, 35 versus 55% of control levels of switching, respectively, which is consistent with an additive effect of the two mutations (Fig. 2, F and G). We conclude that phosphorylation of S38 and T140 are important for class switching in LPS- and IL-4–stimulated B cells. Furthermore, higher levels of AID expression drive higher levels of switching to IgG1 and diminish the requirement for S38 or T140 phosphorylation. Therefore, the precise contribution of these posttranslational modifications to AID function is difficult to evaluate in this assay.

To determine if AID protein levels are a rate-limiting factor in CSR, we augmented AID levels in wild-type LPS- and IL-4–stimulated B cells with retrovirally expressed AID and measured isotype switching to IgG1. Compared with wild type alone or wild type transduced with empty PMX retrovirus, cells supplemented with AID from the PMX-AID retrovirus displayed a >40% increase in isotype switching (Fig. 2 J). We conclude that AID levels are rate limiting and that superphysiological levels can drive higher rates of isotype switching.

To examine the physiological function of AID phosphorylation at positions S38 and T140, we produced mice that carry S38A or T140A mutations in AID, AID-S38A, and AID-T140A, respectively (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20081319/DC1). AID-S38A and AID-T140A mutant B cells developed normally (not depicted) and were assayed for CSR to IgG1 after stimulation with LPS and IL-4 in culture. Cell division was monitored by labeling cells with CFSE and tracking dye dilution by flow cytometry. AID protein levels expressed by wild-type, AID-S38A, and AID-T140A B cells were indistinguishable when measured by Western blotting (Fig. 3 A), and heterozygous AID 4/−, AID-S38A/−, and AID-T140A/− expressed half as much AID as their homozygous counterparts (Fig. 3 A). AID-S38A and AID-T140A B cells divided normally in response to LPS and IL-4, but were impaired in switch recombination to IgG1 (Fig. 3, B and C). AID-S38A mutant B cells showed 32% of the level of CSR to IgG1 of wild-type controls, whereas AID-T140A produced a milder defect resulting in 87% of wild-type CSR (Fig. 3, B and C). In both cases, the defect was exacerbated in haploinsufficient mice. Although AID 4/− B cells displayed IgG1 switching, 80% of wild-type B cells, AID-S38A/−, and AID-T140A/− showed 4 and 45% the level of wild-type B cells, respectively (Fig. 3, B and C). This relative decrease was consistent within each experiment, despite overall switching rates varying from experiment to experiment (Fig. 3, B and C). We also measured mutation within the region 5′ of the Sµ switch repeats. Similar to CSR, AID-S38A and AID-T140A had mutation rates of 25 and 85%, respectively, compared with WT (Fig. 3 D). Because different stimulation conditions may result in differential levels of AID phosphorylation (Fig. 1 C), we measured isotype switching to IgG3. After stimulation with LPS or LPS and anti-dextran in culture, AID-S38A B cells showed 19 or 16% the level of CSR to IgG3 of wild-type controls, whereas AID-T140A produced a milder defect resulting in 71 or 52% wild-type CSR (Fig. 3, E and F). We conclude that both AID-S38 and -T140 phosphorylation are required for physiological levels of CSR, but neither is essential for this reaction.

To examine the role of AID phosphorylation in SHM, we cloned and sequenced the DNA region downstream of IgJ H 4 from purified lymph node GC B cells (46). The effect of AID-S38A on somatic mutation was similar to CSR, resulting in 30% of wild-type activity (Fig. 4 A). In contrast, AID-T140A had more profound effects on somatic mutation than CSR, resulting in 45% of wild-type activity, which was not significantly different from AID-S38A (Fig. 4 A). Similar results were obtained from Peyer’s patch B cells, where mutation rates for AID-S38A and AID-T140A were 20 and 40% of controls, respectively (Fig. 4 B). Haploinsufficiency by itself resulted in a mild decrease in hypermutation with 70% hypermutation activity in AID 4/− versus wild type (Fig. 4 A). Haploinsufficiency magnified the defect in hypermutation of AID-S38A/− and AID-T140A/−, with neither statistically rising above the background levels of mutation.
Figure 2. Comparison of AID, AID<sub>T140A</sub>, and AID<sub>S38A</sub> hypermutation and CSR activity in B and non-B cells. (A) The graph shows a log plot of numbers of kanamycin-resistant (Kan<sup>R</sup>) colonies after induction of AID, AID<sub>S38A</sub>, AID<sub>T140A</sub>, AID<sub>ST-AA</sub>, or empty vector expression. (B) Anti-p140, -p38, or -AID immunoblot of FLAG-tagged AID, AID<sub>S38A</sub>, or AID<sub>T140A</sub> purified from B cells stimulated with LPS and IL-4. (C) Accumulation of GFP-expressing 3T3-NTZ cells after transduction with AID-, AID<sub>S38A</sub>-, or AID<sub>T140A</sub>-expressing PMX-MK retroviruses. 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. (D) Number of mutations in the GFP gene cloned from 3T3-NTZ cells 11 d after transduction with retroviruses encoding AID, AID<sub>S38A</sub>, AID<sub>T140A</sub>, or 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 Student's t test assuming unequal variance and comparing AID expressing with AID<sub>S38A</sub>- and AID<sub>T140A</sub>-expressing cells. P values are indicated. The numbers of point mutations were as follows: 4 mutations/21,643 bp mutations for vector; 59 mutations/13,174 bp for AID; 15 mutations/19,761 bp for AID<sub>S38A</sub>; and 45 mutations/21,643 bp for AID<sub>T140A</sub>. (E) Schematic of retroviral constructs used (top); anti-AID immunoblot of AID<sub>S38A</sub>-B cells infected with PMX-AID, or -AID<sub>S38A</sub>, or -AID<sub>T140A</sub>, or -AID<sub>ST-AA</sub> (middle); or wild-type B cells (WT) or AID<sup>T140A</sup>-B cells infected with PMX-AID, MK-AID, or -AID<sub>S38A</sub>, or -AID<sub>T140A</sub>, or -AID<sub>ST-AA</sub> (bottom). Anti-tubulin was used as a loading control. (F) Graph of flow cytometric analysis of IgG1 expression in AID<sup>T140A</sup>-B cells transduced with PMX-AID, -AID<sub>S38A</sub>, -AID<sub>T140A</sub>, -AID<sub>ST-AA</sub>, or control vector and cultured in LPS plus IL-4 for 3 d after transduction. The percentage of IgG1<sup>+</sup> cells is indicated for five independent experiments, and the mean is indicated by a solid line.
found in AID−/− lymph node GC B cells (Fig. 4 A). Similar, but slightly less pronounced, effects were found in chronically stimulated Peyer’s patch GC B cells (Fig. 4 B). We conclude that both AID-S38 and -T140 phosphorylation are required for optimal somatic mutation and that the T140 has a more profound effect on this reaction than on CSR (Fig. 4 C).

AID-deficient mice and humans have large GCs compared with controls (12, 14). To determine whether this effect is caused by loss of AID protein or its activity, we measured the number of GC B cells in AID−/−, AID+/−, AID140A−, AID38A−, and wild-type mice (Fig. 5, A and B). We found that the amount of AID activity was inversely proportional to the size of the GC response. AID−/− mice showed the highest number of GC B cells, wild-type mice the fewest, and AID+/− haploinsufficient mice were intermediate between the two (Fig. 5, A and B). Decreasing AID activity, but not protein, in AID38A−/− mice resulted in an increase in the number of GC B cells proportional with the relative decrease in activity when compared with AID+/− controls (Fig. 5, A and B). We conclude that the number of GC B cells in immunized mice is inversely proportional to the amount of AID activity.

**DISCUSSION**

Mice and humans deficient in AID have larger GCs than controls (12, 14). In this study, we observe larger GCs in AID-haploinsufficient mice together with further enlargement in AID38A−/− mice, indicating that the GC phenotype is directly related to the level of AID activity. AID activity might impact GC size by a variety of mechanisms, including antibody gene or generalized genomic damage. For example, increased AID activity could result in higher rates of detrimental antibody gene mutation, leading to loss of affinity, loss of expression, or development of self-reactive antibodies (3, 4). Conversely, higher rates of off-target genomic damage by AID may lead to mutations that result in cell death (47). Irrespective of the mechanism, the magnitude of the effect of AID levels on GC size emphasizes the importance of AID regulation in vivo.

Phosphorylation of AID at position S38 is believed to facilitate the interaction between AID and RPA, a cofactor that is required for AID to access transcribed DNA in vitro (37). RPA binds to ssDNA and it was proposed that RPA might stabilize ssDNA liberated during transcription to serve as a substrate for AID (48). Consistent with this idea, AID-S38A is inactive on transcribed dsDNA in the absence of RPA, while retaining catalytic activity on ssDNA (37). Nevertheless, AID-S38A is active at between 20 and 35% of wild-type levels in hypermutation and CSR in vivo. One way to reconcile these results would be to posit that association between AID and RPA is only required for a subset of AID substrates that do not stabilize ss regions spontaneously. For example, ssDNA in switch regions is stabilized spontaneously by R-loops, but this does not occur in Ig variable region DNA. This model might predict that the S38A mutation would preferentially interfere with Ig variable region hypermutation (49, 50). However, the AID38A mutation affects switching and mutation equally; therefore, the mechanism by which S38 phosphorylation alters AID function is likely to be independent of whether the ssDNA substrate is created by R-loops. An alternative nonexclusive explanation for our results is that S38 phosphorylation enhances AID activity by facilitating its interaction with chromatin (29).

Biochemical studies with AID purified from insect cells assayed on artificial substrates showed that S38A mutation does not alter overall AID activity, but shifts target preference in a way that might result in specific loss of activity on hotspots found in the Ig switch region (39). This in vitro bias is not consistent with our mutation data in transfected fibroblasts or GC B cells because we find lower levels of SHM in all cases and the selective hotspot bias found in the in vitro transcription systems would not affect the rate of SHM (29).

Previous communoprecipitation and in vitro kinase experiments suggest that PKA phosphorylates AID at S38; the importance of this kinase in regulating AID is supported by the finding that inhibition of its activity in B cells inhibits class switching (37, 38). Our experiments indicate that T140 is not a PKA target site, and that S38 can be phosphorylated by either PKA or PKC in vitro (Fig. 1 E). In addition, PKC family members overlap in their preference to phosphorylate T140 or S38 in vitro (Fig. 1 F) and S38 or T140 phosphorylation is nonexclusive (Fig. 2 B). Thus, AID activity may be modulated by phosphorylation on one or both sites, depending on which kinase is activated. The PKC family is composed of at least 10 serine/threonine kinases, many of which are expressed in activated B cells (51, 52). They have been implicated in a wide variety of cellular processes, including growth, differentiation, tolerance, immunity, and tumor development (51). Which family members phosphorylate AID and the precise pathways that regulate phosphorylation in vivo remains to be determined.

How T140 phosphorylation modulates AID activity is not known, but this modification differs from S38 in that it preferentially affects somatic mutation. Differential regulation...
Figure 3. CSR in AID^{S38A} and AID^{T140A} mice. (A) Anti-AID immunoblot of AID^{-/-}, AID^{+/-}, AID^{T140A/-}, AID^{S38A/-}, wild-type (WT), AID^{T140A/-}, and AID^{S38A/-} B cells stimulated with LPS and IL-4. Anti-tubulin immunoblot was used as a loading control. (B) Flow cytometric analysis of IgG1 expression and CFSE dye dilution by WT, AID^{-/-}, AID^{T140A/-} and AID^{S38A/-} (top) or AID^{+/-}, AID^{T140A/-}, or AID^{S38A/-} B cells (bottom) stimulated with LPS and IL-4. The percentage of IgG1+ cells is indicated on the top right of each graph. (C) Isotype switching to IgG1 by WT, AID^{T140A/-}, and AID^{S38A/-} (top) or AID^{+/-}, AID^{S38A/-}, and AID^{T140A/-} cells (bottom) as in B for the indicated number of independent experiments. Solid lines represent means. Bar graphs represent the mean relative efficiency and SD of IgG1 CSR compared with WT. (D) Number of mutations in the 5' of Sμ region cloned from AID^{S38A/-}, WT, AID^{T140A/-}, and AID^{S38A/-} B cells stimulated with LPS and IL-4 sorted for IgM expression and five cell divisions. Pie charts and statistical analysis, as in Fig. 2 D, represent summary of two experiments. The numbers of point mutations were as follows: 3 mutations/59,185 bp mutations for AID^{S38A/-}; 18 mutations/77,875 bp for WT; 11 mutations/115,255 bp for AID^{S38A/-}; and 24 mutations/117,747 bp for AID^{T140A/-}. (E) Flow cytometric analysis of IgG3 expression in AID^{-/-}, WT, AID^{T140A/-}, and AID^{S38A/-} B cells stimulated with LPS or LPS and anti-dextran for 6 d. The percentage of IgG3+ cell is indicated on the top left of each graph. (F) Results of E from three independent experiments. Solid line represents the mean.
of class switching and somatic mutation is a well-characterized physiological feature of gene diversification in B cells. For example, B cells undergoing switch recombination in response to LPS and IL-4 in vitro do not mutate their Ig variable regions. This cannot be ascribed to a general absence of the factors that mediate hypermutation because the DNA of the I/H9262 promoter and the switch regions themselves are mutated by AID in LPS- and IL-4–stimulated B cells. Indeed, the amount of mutation in switch regions in AID-T140A–expressing B cells is directly proportional to the amount of class switching and differs from Ig variable region gene mutation, which is more severely affected (Fig. 4 C). One explanation for the disparity between the effects of AID-T140A on switching and mutation would be that there are specific requirements for AID in the two reactions. This idea is supported by the finding that mutations in the amino or carboxyl terminal regions of AID preferentially affect somatic mutation or class switching (45, 53).

Finding that phosphorylation at T140 preferentially affects somatic mutation suggests a mechanism by which AID activity on closely related substrates might be differentially regulated.

Figure 4.  SHM in GC B cells from AIDS38A and AIDT140A mice. (A) GC B cells were purified from the lymph nodes of 5 immunized AID−/−, AID+/−, AIDT140A−/−, AIDS38A−/−, WT, AIDT140A, or AIDS38A mice. Pie charts indicated the number of mutations in the intronic region of JH4. Pie charts and statistical analysis as in 2D. The numbers of point mutations were as follows: 2 mutations/33,666 bp mutations for AID−/−; 184 mutations/115,116 bp for WT; 41 mutations/96,654 bp for AIDS38A; and 65 mutations/85,794 bp for AIDT140A (top). 2 mutations/43,983 bp mutations for AID−/−; 227 mutations/45,612 bp for WT; 33 mutations/46,698 bp for AID−/−; 51 mutations/66,246 bp for AIDS38A−/−; 8 mutations/71,676 bp for AIDT140A−/− (bottom). (B) GC B cells purified from Peyer’s patch analyzed the same as A. The numbers of point mutations were as follows: 2 mutations/33,666 bp mutations for AID−/−; 41 mutations/96,654 bp for WT; 4 mutations/66,246 bp for AIDS38A−/−; and 8 mutations/71,676 bp for AIDT140A−/− (bottom). (C) Summary of efficiency of both SHM and isotype switching to IgG1 relative to wild-type by AIDT140A−/−, AIDS38A−/−, AIDT140A, and AIDS38A/AID−/− cells. Bars represent the means from and statistics are reported in Figs. 3 C, 3 D, and 4 (A and B).
MATERIALS AND METHODS

Mice. To produce AID<sup>S38A</sup> and AID<sup>T140A</sup> mice, AID S38 or T140 was mutated to alanine using the same gene-targeting strategy (Fig. S1). The long arm of the targeting vector was 5 kb long with 3′ within the intron between AID exons 2 and 3 (Fig. S1). The short arm was a 3.3-kb fragment extending with the 3′ into the intron between exons 3 and 4. A LoxP-flanked neomycin-resistance gene was used for positive selection, and a diphtheria toxin gene was used for negative selection. The targeting construct was linearized and transfected into C57BL/6 embryonic stem cells. The genotype was confirmed by Southern blot and PCR amplification using a primer outside of and transfected into C57BL/6 embryonic stem cells. The genotype was confirmed by Southern blot and PCR amplification using a primer outside of and transfected into C57BL/6 embryonic stem cells. The genotype was confirmed by Southern blot and PCR amplification using a primer outside of and transfected into C57BL/6 embryonic stem cells. The genotype was confirmed by Southern blot and PCR amplification using a primer outside of and transfected into C57BL/6 embryonic stem cells.

Lymphocyte isolation and culturing and retroviral infection. Lymphocyte isolation, cultures, retrovirus infection, and analysis were previously described (29). The PMX-MK-AID was the same as the previously described PMX-AID, except the Kozak CCACCATGG was changed to GTTGTATGG. B cells were purified from mouse spleens by depletion with anti-CD43 beads (Miltenyi Biotec) and cultured in RPMI medium, 10% FBS, 5 ng/ml IL-4 (Sigma-Aldrich), and 25 μg/ml LPS (Sigma-Aldrich) for 72 h (29).

PCR and mutation analysis. For SHM analysis, age-matched 8- to 16-wk-old mice were immunized with footpad injection with 50 μg of NP-CGG (Biosearch Technologies) precipitated in alum and lymph node, and Peyer’s patches were dissected 11 d after immunization. GC B cells were stained with APC anti-CD19, FITC anti-GL7, and PE anti-FAS antibodies (BD Biosciences) and purified by cell sorting. In two independent experiments, DNA samples were pooled from 2 or 3 separate mice. To amplify the intrinsic region 3′ of JH4, four separate PCR reactions were performed on each sample with Pfu polymerase (Stratagene) on genomic DNA from 5,000 equivalent cells. Amplified products were pooled, cloned, and sequenced. The intron region 3′ of JH4 was amplified with a common V<sub>δ</sub>2J<sub>δ</sub>58 family primer (5′-GGAAATTCGGCTGACATCTGAGGACTCTGC-3′) and (5′-CTGGACTTTTCGGTTGTGTCG-3′) for 9 cycles at 94°C (30 s), at 55°C (30 s), and at 72°C (90 s), and then (5′-GGTGCAAGGAACCTCAGTCA-3′) and (5′-TCTGCTAGACACAGCTAC-3′) for 21 cycles at 94°C (30 s), at 55°C (30 s), and at 72°C (30 s). For 5′ microarray region analysis after 96-h LPS/IL-4 stimulation, IgM<sup>+</sup> cells from two mice that CSFE labeling indicated had divided 5 times were sorted. Conditions and primers were previously described (29). The NTZ-3T3 assay and GFP gene mutational analyses were performed as previously described 11 d after retrovirus infection (33, 44). PCR products were cloned into TOPO-TA cloning kit (Invitrogen) and sequenced with T7 primer. E. coli assays were performed exactly as previously described (19).

Protein analysis. Anti-AID and -p38 antibodies were previously described (29). To produce anti-p140 antibodies, rabbits were immunized with phosphopeptide GVQIGIM(pT)FKDYFYFC (AID 133–148) coupled to keyhole limpet hemocyanin. Phosphospecific antibodies were purified by negative selection on unphosphorylated peptide AID 133–148 coupled to Sulfolink gel (Thermo Fisher Scientific), followed by positive selection on the phosphopeptide. Cells were extracted in lys buffer (20 μM Tris, pH 8.0, 400 μM NaCl, 1% Nonidet P-40, 0.5 μM EDTA, 25 μM NaF, and 1 mM DTT). For immunoprecipitation, 2 mg of extracts were incubated with anti-AID antibody and protein A-Sepharose (GE Healthcare) for 2 h. For FLAG immunoprecipitation, anti-FLAG agarose beads (Sigma-Aldrich) were incubated with extracts for 2 h and AID was eluted with 0.5 μg/ml of FLAG peptide (Sigma-Aldrich) in lys buffer. Western blots were performed on immunoprecipitated protein or on 50 μg of total cell extracts with the indicated anti-AID antibody; anti-tubulin (Abcam) was used as a loading control. To compare AID levels from retroviral-infected B cells, Western blots were performed on cells sorted for GFP expression. AID purification and mass spectrometry analysis of phosphorylation was performed as previously described on extracts from 10<sup>6</sup> wild-type B cells purified from mouse spleens by depletion with anti-CD43 beads (Miltenyi Biotec) and cultured in RPMI medium, 10% FBS, 5 ng/ml IL-4 (Sigma-Aldrich), and 25 μg/ml LPS (Sigma-Aldrich) for 72 h (29).

In vitro phosphorylation. 100 ng of purified recombinant AID protein was incubated with PKA as previously described (29) or 0.1 U of PKC catalytic subunit or isoforms (Calbiochem) for 30 min at 30°C in a buffer containing 40 mM MES, pH 6.0, 1 mM EGTA, 10 mM MgCl<sub>2</sub>, 1 mM DTT, and 0.1 mM ATP.

Online supplemental material. Fig. S1 shows gene-targeting strategy to produce AID<sup>S38A</sup> and AID<sup>T140A</sup> mice. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20081319/DC1.

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