Altered Pattern of Immunoglobulin Hypermutation in Mice Deficient in Slip-GC Protein

Kathleen Richter†, Laurannel Burch§, Frank Chao§, David Henke§, Chuancang Jiang¶, Janssen Daly†, Ming-Lang Zhao†, Grace Kissling‡, and Marilyn Diaz†

From the †Somatic Hypermutation Group, §Molecular Genetics Core, Laboratory of Molecular Genetics, and ¶Biostatistics Branch, Environmental Diseases and Medicine Program, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709

Background: SLIP-GC is a novel GTPase expressed in germinal center B cells that localizes to sites of DNA replication. Results: B cells from SLIP-GC-deficient mice displayed increased G:C transitions at immunoglobulin and non-immunoglobulin loci. Conclusion: SLIP-GC may protect activated B cells from AID-mediated deamination of cytosines. Significance: SLIP-GC is a novel molecule involved in the prevention or repair of G:C transitions in activated B cells.

Somatic immunoglobulin (Ig) hypermutation (SHM) is the process wherein Ig variable (V) genes are deliberately hypermutated to enhance the affinity of antibodies to a specific antigen (1, 2). It occurs in the germinal centers (GC) of secondary lymphoid tissues such as the spleen and the lymph nodes during an immune response to foreign antigens (3). SHM is coupled to positive selection of B cells that have acquired affinity-enhancing mutations to the foreign antigen, a process known as affinity maturation. Affinity maturation of B cells during immune responses leads to the formation of memory B cells that contribute significantly to a swift, high-affinity memory B cell response upon re-exposure to antigen (4).

SHM is triggered by the activation-induced deaminase (AID), a cytidine deaminase. AID is expressed in activated B lymphocytes, and it deaminates cytosines in the DNA encoding the IgV regions, the gene segments encoding the antigen-interacting portions of antibodies (5, 6). AID-mediated deamination of cytosines in the DNA of IgV regions leads to the formation of uracil opposite guanine bases (7–9), which can be removed by uracil DNA glycosylase to generate an abasic site. Staggered abasic sites in both strands can lead to the formation of DNA breaks. These DNA lesions are the critical step that leads to either SHM or class switch recombination (CSR) (7, 10, 11). In SHM, error-prone DNA synthesis, triggered by the abasic site or the G:U mismatch, leads to mutations throughout V regions (12), whereas in CSR, AID-mediated DNA double strand breaks are required to trigger the reaction, likely through the activation of nonhomologous recombination repair of double-stranded DNA breaks (7, 10).

Because of the potential to generate mutagenic DNA lesions, several layers of negative regulation for AID have evolved (13). AID is regulated at the expression level; only activated plasma cells or GC B cells express it (14). Intracellular localization of AID is also regulated through CRM1-mediated nuclear export (15–17), and microRNAs that reduce AID transcript levels have been described (18–20). However, the targeting mechanism of AID to IgV and switch regions remains elusive. Although no region in the genome of activated B cells mutates to the level of IgV regions, the mechanism is leaky, and AID sometimes deaminates cytosines in actively transcribed non-Ig genes (21). When this involves tumor suppressor genes or oncogenes, untargeted AID activity can lead to the development of B cell lymphomas (22). In addition, AID levels play a role in the development of autoimmunity (23–29). Clearly, although AID is critical to memory B cell immune responses, it is also a dangerous molecule that is normally carefully regulated.

We have recently characterized a novel GC-expressed protein, SLIP-GC (speckled-like pattern in the germinal center),
which appears to protect B cell lymphoma lines from AID-dependent DNA breaks by blocking access of AID to replicating DNA (30). These results lead to the hypothesis that SLIP-GC contributes to the negative regulation of AID-mediated deamination of cytosines in GC B cells. To test this, we generated SLIP-GC-deficient mice and examined Ig hypermutation in Peyer’s patches (PP) B cells of young and old mice. We also examined hypermutation in non-Ig genes known to mutate by AID at very low frequencies. The results show that SLIP-GC deficiency causes an increase in GC transitions at Ig and non-Ig loci that is AID-dependent and strongly suggest a role for this novel protein as a negative regulator of AID activity in GC B cells.

EXPERIMENTAL PROCEDURES

Generation and Genotyping of Slip-GC<sup>−/−</sup> Knock-out Mice—A vector was generated containing loxP sites flanking exons 2 and 4 of the Slip-GC gene. Exon 2 harbors the conserved GTPase domain of SLIP-GC (30) (supplemental Fig. 1). Vector was transfected into C57BL6 ES cells, and germline transmission was obtained. Initially, mice were crossed to Sox2 promoter-driven Cre-recombinase transgenics, which are in a mixed genetic background. Cre-recombinase heterozygous, Slip-GC homozygous floxed mice were backcrossed into Slip-GC homozygous floxed mice in a pure C57BL/6J background for at least six generations to generate Sox2-cre heterozygous, Slip-GC floxed homozygous mice in the C57BL/6J background (supplemental Fig. 1). To confirm wild type (WT), heterozygote, or homozygote for the floxed gene or Slip-GC-deficient (Slip-GC<sup>−/−</sup> cre+) genotypes, PCR was performed with three primers: SlipGC 5′floxed forward, 5′-GAA ATG ACT GGG GTG TCC TGA GGC-3′; GCS P, 5′-Flox reverse (N16ck5R), 5′-GTG CCC GCT TAC CAC AGA ACT TATG-3′; and GCS P 3′Flox reverse (N16del3R2), 5′-GAT AGC TTT GGT GCC AGC ATT CAT C-3′. The WT allele results in a 0.55-kb PCR product, where the Slip-GC mutated allele results in a 0.68-kb PCR-product.

Mice used in these studies were 6–24 weeks of age. All the mice were housed in the animal facility at the NIEHS, National Institutes of Health, under specific-pathogen-free conditions, and animal protocols were approved by the Institutional Animal Care and Use Committee.

RT-PCR Studies on the Expression of Slip-GC and AID—Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. cDNA (20 μl) was synthesized with 1 μg of total RNA as template using SuperScript III first-strand synthesis supermix (Invitrogen) according to the manufacturer’s instructions. Expression levels of AID and Slip-GC were estimated using real-time PCR. The RT-PCR primers were synthesized by Invitrogen: mAID forward, 5′-GGG GAC CGA CAT TAT GGA CAG CCT TCT G-3′; mAID reverse, 5′-TCA AAA TCC CAA CAT ACG AAA TGC-3′; Slip-GC mice exon 2, forward 5′ GAG TGT CGG ACC AGG GTT GTT T-3′; and Slip-GC mice exon 3 reverse, 5′-CAG GGC CAT AGA GCA TCC GTA G-3′. PCR reactions were performed in 50-μl volumes containing 2 μl of cDNA sample, 1 × PCR buffer, 10 mM/liter deoxynucleotide triphosphates mix (dNTPs), 2 mM/liter each sense and antisense primers, and 5 units of Taq polymerase (Invitrogen). After an initial 3-min incubation at 95 °C, 36 PCR cycles were carried out using the following conditions: denaturation at 95 °C for 10 s, annealing at 57 °C (Slip-GC) and 52 °C (AID) for 30 s, and extension at 72 °C for 45 s. To verify that equal amounts of cDNA were added to each PCR reaction, we used the housekeeping gene GAPDH (mGAPDH forward primer 5′-ACC ACA GTG CAT GCC ATC AC-3′ and mGAPDH reverse primer 5′-TCC ACC ACC CTG TTG CTG TA-3′; annealing temperature 52 °C, 27 cycles). PCR products were separated on a 1.5% agarose gel and analyzed.

Mouse Immunization—Mice were injected intraperitoneally with 150 μl of 2,4,6-trinitrophenyl chicken gamma globulin (TNP(24)-CGG) (Biosearch Technologies, Novato, CA) at three different sites.

Germinal Center Staining—Mice were sacrificed, and spleens were taken 5, 7, and 10 days after immunization with TNP(24)-CGG (Biologene Technologies) and fixed quickly in optimum cutting temperature compound (O.C.T. Sakura Finetechnical) using dry ice. Frozen, O.C.T.-embedded tissues were cryosectioned and fixed with Rapid-Fix fixative (Richard-Allen Scientific, Kalamazoo, MI) for 7 s, and endogenous peroxide was blocked with 0.3% hydrogen peroxide. Protein blocking was done with the avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA). Primary antibody, peanut agglutinin (Vector Laboratories), diluted with 1 μg/ml CaCl<sub>2</sub> and MgCl<sub>2</sub>, MnCl<sub>2</sub> was incubated for 60 min at 1:1000 dilution. Slides were developed with diaminobenzidine, counterstained with hematoxylin, dehydrated, cleared, and coverslipped.

Somatic Hypermutation in Peyer’s Patch B Cells—PP were collected from mice at 8, 12, 16, and 24 weeks of age, and single-cell suspensions were made by squashing Peyer’s patches between two frosted slides. After washing in buffer (PBS, 0.1% sodium azide, 1/100 l-glutamine), cells were stained with conjugated antibodies: phycoerythrin-conjugated anti-B220 (0.2 μg per 10<sup>6</sup> cells), phycoerythrin-Cy7-conjugated anti-CD19 (0.2 μg per 10<sup>6</sup> cells), and fluorescein isothiocyanate-conjugated anti-GL7 (0.5 μg per 10<sup>6</sup> cells) (BD Biosciences). B220<sup>−</sup>CD19<sup>−</sup>GL7<sup>+</sup> cells were sorted using a BD Biosciences FACS Vantage SE flow cytometer.

The IgH variable region DNA was amplified by PCR. Briefly, for the IgH locus, a 1.2-kb fragment from the intronic region 3′ of the rearranged endogenous VH genes was amplified using a primer VHJ558 forward (GCC TGA CAT CTG AGG AGT CTG C) along with a primer IgH intronic enhancer reverse (CCT CTC CAG TTT CGG CTG AAT CC) with Phusion DNA polymerase (New England Biolabs, Ipswich, MA). The PCR was performed at 98 °C for 3 min; 35 cycles of 98 °C for 10 s, 70 °C for 30 s, and 72 °C for 40 s; and 72 °C for 6 min. The PCR products were electrophoresed through a 1% agarose gel (SeaKem≤LE agarose, Lonza Rockland, Rockland, ME). A bright 1.2-kb band was excised and purified using a QIAquick<sup>®</sup> gel extraction kit (Qiagen). The 1.2-kb DNA fragments were cloned using a TOPO TA Cloning<sup>®</sup> pCR<sup>®</sup>2.1-TOPO<sup>®</sup> kit (Invitrogen) following the manufacturer’s instructions. Shot<sup>®</sup> Top10 chemically competent Escherichia coli (Invitrogen) were transformed with the TOPO vector, spread on Luria-Bertani broth (LB) agar (ampicillin 100 μg/ml) plates, which had been
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spread with 2% X-gal and 0.1 m isopropyl-1-thio-β-d-galactopyranoside, and incubated at 37 °C for 16 h. Plasmid DNA was prepared from positive white colonies, and the inserted VH 1.2-kb PCR fragments were sequenced with a primer specific to the region immediately downstream of JH4 (TAT GCT ATG GAC TAC TGG) or M13 forward (~20) primer (GTA AAA CGA CCG CCA G) and M13 reverse primer (CAG GAA ACA GTG ATG AC).

Non-Ig Gene Mutational Analysis—Seven B6 control and five SLIP-GC-deficient mice, which were 4 months of age, were used for this analysis. Positive germinal center Peyer’s patch B cells were isolated by FACS after staining with conjugated antibodies: phycoerythrin-conjugated anti-B220, phycoerythrin-Cy7-conjugated anti-CD19, and fluorescein isothiocyanate-conjugated anti-GL7. Genomic DNA was isolated as described above. The following primers were used to amplify specific genes from the mouse genome by PCR followed by analyzing by sequencing: CD79b/H23041 Top10 chemically competent cells were spread with 2% X-gal and 0.1M isopropyl-1-thio-D-galactopyranoside, and incubated at 37 °C for 16 h on LB ampicillin plates, which formed into One Shot products were cloned into pCR manual. After adding A overhangs to their blunt ends, PCR products (98 °C for 10 s, 64 °C for 10 s and 72 °C for 40 s; and 72 °C for 6 min. PCR products (36 cycles of 98 °C for 10 s, 64 °C for 10 s and 72 °C for 40 s; and 72 °C for 6 min. PCR products (CD79b/H23041 forward, CD79b/H23041 reverse) primer (GTA AAA GAT CCG CCA G) and M13 reverse primer (CAG GAA ACA GTG ATG AC).

Class Switch Recombination Assay—Resting/naive splenic B cells were isolated by using CD43 (Ly-48) MicroBeads following the manufacturer’s instructions (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). Ammonium chloride-potassium lysing buffer (0.15 mM NaCl, 10.0 mM KHCO3, 0.1 mM Na2EDTA, pH 7.4) was used to eliminate red blood cells. CD43-isolated B cells were incubated at 1 × 106 cells/ml in complete B cell culture medium (Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 1% nonessential amino acids, 2 mM l-glutamine, 50 μM 2-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin sul fate, 10 mM HEPES, pH 7.4, all from Invitrogen) and stimulated in vitro with or without LPS (LPS; 50 μg/ml), LPS + IL-4 (LPS; 50 μg/ml and IL-4; 20 ng/ml), or LPS + IFN-γ (LPS; 50 μg/ml and mIFN-γ; 20 ng/ml) for 96 h. LPS was from Sigma, and cytokines were from R&D Systems (Minneapolis, MN). To detect mouse IgM, total IgG, IgG3, IgG1, IgG2b, IgG2c, and IgA supernatant was collected, diluted (IgG3 (1:4), IgG1 (1:2), IgG2c (no dilution)), and analyzed by using enzyme-linked immunosorbent assay (ELISA) kits from Bethyl Laboratories (Montgomery, TX) following the manufacturer’s instructions.

B Cell Development—Bone marrow cells from two mice of age 6 and 8 weeks were prepared by flushing femurs or, in some experiments, both femurs and tibias with FACS buffer (Hanks’ balanced salt solution (Invitrogen) supplemented with 3% FCS (Sigma) and 10 mM HEPES (Invitrogen)) using a syringe and a 23-gauge needle. The cells were centrifuged at 1200 rpm (319 × g) for 3 min at 4 °C. The supernatant was removed, and the cell pellet was gently resuspended in FACS buffer and centrifuged as described above. Finally, after resuspending the pellet in FACS buffer, the cells were filtered through 30-μm nylon mesh or, if subsequently being used in tissue culture, 40-μm sterile nylon filters (BD Falcon). Viable, nucleated cells were counted using a 0.4% aqueous trypan blue solution (Sigma) exclusion and adjusted to the appropriate concentration.

Spleen cell suspensions were prepared by gently squeezing pieces of spleen between two microscope slides with frosted ends in the presence of FACS buffer. Cells were filtered through a 40-μm sterile nylon filter, washed twice in FACS buffer (centrifugation as described above), and finally resuspended in FACS buffer.

0.5 × 106 cells of either cell solution were stained in alternate wells of a round-bottomed 96-well plate (Nunc) with the following antibodies: fluorescein isothiocyanate (FITC)-labeled rat anti-mouse B220/CD45R (clone RA3-6B2); phycoerythin-
labeled rat anti-mouse-B220/CD45R (clone RA3-6B2), IgM (cloneR6-60.2), CD25/IL-2Rα-chain (clone Pc61), CD117/c-Kit (clone ack45) and CD19 (1D3); allophycocyanin-labeled rat anti-mouse IgG1, IgG2a, and IgG2c light chain (clone R26-46) (Pharmingen). Allophycocyanin-labeled anti-mouse Ly-77 (clone GL7) was from eBioscience. Biotinylated antibodies were revealed using streptavidin-phycocerythrin (Southern Biotechnology Associates), streptavidin-allophycocyanin (Pharmingen), or streptavidin TRI-COLOR (Caltag Laboratories). After centrifugation (1200 rpm for 2 min at 4 °C), the cells were resuspended and incubated (25 μl final volume) with the appropriate combinations of the above antibodies at optimal dilutions in FACS buffer on ice for 30 min in the dark. The cells were washed and, when necessary, biotinylated antibodies were revealed by incubation for a further 15 min on ice with streptavidin conjugated with appropriate fluorochrome. The cells were resuspended in FACS buffer and analyzed with a FACSCalibur™ with Consort 30 software (Becton Dickinson) equipped with the appropriate filters for four-color immunofluorescence. Dead cells were visualized by adding propidium iodide (0.25 μg/ml final concentration (Sigma) prior to acquisition. Typically, 10,000 live, nucleated events were collected. Data were analyzed using Flowjo software (Ashland, OR).

Statistics—Fisher’s exact test was used to compare mutation frequencies. One-sided p values were used when the direction of the difference could be predicted a priori; otherwise, two-sided p values were used. All probability values were considered significant if less than 0.05.

RESULTS

To examine the role of the novel GTPase, SLIP-GC, in SHM and in CSR, we generated mice with loxP regions flanking three exons within the gene encoding this protein (Fig. 1A; supplemental Fig. 1). One of these, exon 2, harbors the highly conserved GTPase domain (30). These mice were then crossed to Sox2-driven Cre-recombinase transgenics to generate mice deficient in SLIP-GC. These mice completely lacked mRNA transcripts for SLIP-GC, confirming the deletion of the appropriate region (Fig. 1B). The accumulation of mutations in the intronic region downstream of rearranged Ig heavy chain V genes in B cells from PPs was examined by sequencing the JH4 downstream region (described in GenBank™ accession record number NG_005838.1 at 180,260–180,834) (35, 36). This region is subjected to SHM by the same machinery that mutates the IgV region with a frequency of 0.01–0.015 mutations per base pair (35, 36). Because the region analyzed is noncoding, mutations here are a more accurate representation of the intrinsic SHM machinery in the absence of selection. CSR was examined following activation of B cells in vitro with LPS and various cytokines. Finally, we examined mutations in the non-Ig genes, Cd79b and Cd83, previously shown to accumulate a very low mutation frequency (∼10⁻⁵) that is AID-dependent in activated B cells (21).

As in Lymphoma Cell Lines, Transfected SLIP-GC Forms Nuclear Speckles in SLIP-GC-deficient Primary B Lymphocytes—Previously, we observed that transfected SLIP-GC localizes to replication factories and DAPI-stained DNA in fibroblast and lymphoma cell lines such as Ramos and Raji (30) (supplemental Fig. 2A). To determine whether primary B lymphocytes display the same pattern, we transfected SLIP-GC-deficient B cells with a GFP-SLIP-GC expression vector and examined localization of the protein by confocal microscopy. Similarly to the cell lines, SLIP-GC formed nuclear speckles that localized to DAPI-stained regions (supplemental Fig. 2B). Despite its localization to DNA and specifically to replication factories, SLIP-GC deficiency did not alter proliferation of Peyer’s patches or splenic B cells following activation with LPS (supplemental Fig. 2C and D). Apoptosis also appeared unaffected with SLIP-GC deletion in either PP (85% versus 76% viable cells in culture) or splenic B cells following activation with LPS (supplemental Fig. 2E). Previously, we have shown that ectopic expression of SLIP-GC in primary fibroblasts altered neither cell cycle nor proliferation, yet SLIP-GC co-localizes with newly synthesized DNA as revealed by BrdU staining (30).

B Cell Development, Germinal Center Formation, and CSR Are Normal in SLIP-GC-deficient Mice—Impaired SLIP-GC activity did not impact B cell development in the bone marrow or the spleen when compared with C57BL/6J controls. All B cell subsets in the bone marrow and the spleen were present in numbers similar to controls (Fig. 2, A and B). In addition, T cell development was not affected in these mice (supplemental Fig. 3A). Following immunization, SLIP-GC-deficient mice formed normal GCs that appeared abundant (Fig. 2C). In addition, these mice displayed normal or slightly elevated numbers of GL7+ GC B cells (Fig. 2D) in PP GCs. Finally, activated B cells with LPS alone or with IL4 or interferon γ underwent CSR normally as determined by normal levels of IgG1, IgG2c, and IgG3 (Fig. 3, supplemental Fig. 3B). These results are not consistent with SLIP-GC having a crucial role in CSR, and they are consistent with the previous result, suggesting the absence of a proliferation defect with SLIP-GC deficiency because CSR is linked to numbers of cell divisions (37).
Altered Pattern and Frequency of Ig Hypermutation in PP B Cells from SLIP-GC-deficient Mice—GC B cells from PPs were isolated from SLIP-GC-deficient and WT mice ranging in age from 2 through 6 months, and mutation in the JH4 downstream region was examined. There was an increase in the number of mutations per base pair in 3-, 4-, and 6-month-old SLIP-GC-deficient mice, suggesting that these mice accumulated mutations at a higher rate than did C57BL/6J controls in chronically stimulated PP B cells (Fig. 4A). The distribution of mutations was similar between both groups, but B cells from SLIP-GC-deficient mice had a higher percentage of clones with a high number of mutations (i.e., more than 10 mutations within a 426-bp region; Fig. 4B). The increased mutation is mostly at G:C base pairs as B cells from SLIP-GC-deficient mice from each of the age groups had a higher frequency of mutations at G:C base pairs (Fig. 4C). This increase in G:C mutation is the result of a highly significant increase in G to A and C to T transitions when compared with less than 30% of G:C mutations in C57BL/6J mice (Fig. 5). Interestingly, the overall frequency of clones with no mutations was 12% in the controls but only 5% in SLIP-GC-deficient mice, a difference that was not statistically significant but that may indicate a trend. In summary, SLIP-GC-deficient mice experienced an increase in mutation frequency at JH4 downstream regions that was mostly due to a specific increase in G:C transitions at AID hotspots.

SLIP-GC Deficiency Correlated with an Increase in G:C Transitions in the Cd79b and Cd83 Genes of PP B Cells—Given our previous findings suggesting that SLIP-GC protects replicating DNA from AID-mediated deamination of cytosines, we examined mutation at non-Ig loci known to be targeted by AID in activated B cells but at a much lower rate than Ig loci: Cd79b and Cd83 (21). The results were similar to those seen in the JH4 downstream region of the Ig locus. There was a modest increase in the mutation frequency at both genes, and over 90% of G:C mutations in C57BL/6J mice were G to A and C to T transitions when compared with less than 30% of G:C mutation in control mice (Fig. 6). In addition, 30% of G:C mutations in Cd79b and 20% in Cd83 fell in AID hotspots when compared with 0 and 8%, respectively, in control mice. Thus, as seen for
JH4 downstream regions, SLIP-GC deficiency caused an increase in G:C transitions that were more likely to fall in AID hotspots. Combined, these results are most compatible with a model wherein SLIP-GC blocks AID-mediated deamination of cytosines in activated B cells.

**DISCUSSION**

SLIP-GC deficiency significantly altered the pattern of mutations at the IgH J-C intronic region downstream of JH4. This is evident from an overall increase in mutation frequency that is the result of increased G:C mutations, particularly at known AID hotspots. In addition, the mutations at G:C base pairs tended to favor transitions in SLIP-GC-deficient mice, suggesting that the increase in mutation frequency occurred as a result of replication over AID-generated uracils, although the more substantial increase in overall G:C mutation also implicated increased transversions from abasic sites during SHM. These results can be explained by several models including one wherein SLIP-GC acts as a negative regulator of AID activity in SHM but not CSR. A specific effect on SHM, but not CSR, may reflect the fact that CSR and SHM may occur at different stages of the cell cycle. Another possible model suggests SLIP-GC plays a role in the processing or repair of AID-generated uracils, presumably during DNA replication.

We have previously shown that SLIP-GC localizes to replicating DNA in lymphoma cell lines and in fibroblasts transfected with GFP-SLIP-GC (30). Herein, we show that SLIP-GC also forms nuclear speckles that localize to DAPI-stained DNA regions in primary B lymphocytes. Nevertheless, SLIP-GC deficiency did not alter proliferation, and these mice had normal to large germinal centers and, if anything, had increased numbers of germinal center B cells. Despite its co-localization with BrdU-stained DNA (i.e. newly synthesized DNA), ectopic expression of SLIP-GC in fibroblast cell lines did not alter cell cycle dynamics (30). However, ShRNA against SLIP-GC resulted in an increased incidence of AID-mediated DNA breaks in lymphoma cell lines, an apoptosis of those cells (30). These results suggest that SLIP-GC does not play a role in normal replication but may impede AID from targeting and deaminating cytosines in replicating DNA in activated B cells or may alter the repair of the AID-generated uracils during SHM.

**FIGURE 3.** Slip-GC is not necessary for class switch recombination. Splenic B cells from two mice per strain at age 8 weeks were isolated and stimulated in vitro with LPS, IL4, and IFN-γ for 4 days. A–C, IgG1 (A), IgG2c (B), and IgG3 (C) production in splenic naive B cells in SLIP-GC-deficient mice was similar to C57BL/6J mice. Standard deviations are depicted.

Paradoxically, although SLIP-GC deficiency in cell lines results in increased DNA breaks that lead to cell death, SLIP-GC deficiency in mice did not result in smaller or impaired germinal centers. We are currently examining the possibilities that additional factors are present in primary B cells but not in lymphoma cell lines that minimize the apoptotic signals elicited by AID-mediated DNA break or that there is better repair of the DNA breaks in the primary cells. One possibility is that p53 activation by double-stranded DNA breaks is temporally suspended in germinal center B cells while the cells are undergoing SHM and CSR, both processes that can lead to DNA breaks.
It was possible that SLIP-GC deficiency in activated B cells may lead to an increase in G:C transitions not only at Ig loci but also at non-Ig loci. To test this, we examined mutation at Cd79b and Cd83 in PP B cells from 4-month-old mice. Cd79b, and particularly Cd83, have been shown previously to undergo higher than background rates of mutation in activated B cells, and the mutations appear to be partially AID-dependent (21).

In Cd79b, there was only a small increase in mutation frequency in the SLIP-GC-deficient mice. The pattern was markedly different between the strains; close to 100% of mutations from G:C base pairs were transitions in SLIP-GC-deficient mice when compared with less than 40% of G:C mutations in control mice. In addition, G:C mutations in SLIP-GC-deficient mice were more likely to fall within AID hotspots than G:C mutations in control mice. These results are consistent with a role for SLIP-GC as a negative regulator of AID activity during DNA replication or a role in the repair of AID-generated uracils.

**FIGURE 4.** Slip-GC deficiency alters the somatic hypermutation spectrum within the IgH chain intronic region downstream of JH4 of PP B cells. Somatic hypermutation data were collected and analyzed from: young C57BL/6J mice (n = 12 mice; 2–3 months of age) yielding a total of 67 unique clones; older C57BL/6J mice (n = 16 mice; 4–6 months of age), yielding a total of 51 unique clones; young Slip-GC-deficient mice (n = 8 mice; 2–3 months of age), yielding a total of 47 unique clones; older Slip-GC-deficient mice (n = 8 mice; 5–6 months of age), yielding a total of 51 unique clones (clones not sharing mutations from the same animal). A, increased mutation frequency in Slip-GC-deficient mice when compared with C57BL/6J and at 3, 4, and 6 months of age (p value at 2 months = 0.42, at 3 months = 0.027, at 4 months = 0.0019, and at 6 months = 0.0007; Fisher’s exact test). Significant differences between Slip-GC-deficient mice and controls are marked with an asterisk. B, mutation distribution among mutated clones in C57BL/6J and Slip-GC-deficient mice reveals an increase in the fraction of highly mutated clones from SLIP-GC-deficient mice. Significant differences (p < 0.05, Fisher’s exact test) are marked with an asterisk. C, a trend for increased mutations at G:C base pairs in Slip-GC-deficient mice. D, G:C transitions (G to A, C to T) for all ages were increased with SLIP-GC deficiency and are the likely cause of the increase in mutation frequency. Significant differences (p < 0.05, Fisher’s exact test) between C57BL/6J and Slip-GC-deficient mice are marked with an asterisk.
**FIGURE 6.** Increased G:C transitions at the non-Ig loci $Cd79b$ and $Cd83$ in SLIP-GC-deficient mice. Mutations from sorted B220$^/$H11001, CD19$^/$H11001, and GL-7$^/$H11001 B cells from PPs were analyzed in 12 2–4-month-old C57BL/6J mice and in seven 2–4-month-old SLIP-GC-deficient mice. Almost all G:C mutations from SLIP-GC-deficient mice were transitions in $Cd79b$ and $Cd83$. p values are by Fisher’s exact test.

**FIGURE 5.** G:C mutations in B cells from $Slip-GC$-deficient mice were more likely to fall within AID hotspots than G:C mutations from control mice. A, JH4 downstream region (accession NG_005838.1 in the 180,260–180,834 segment) displaying AID hotspots (marked in yellow and numbered 1–26). B, G:C mutation distribution among AID hotspots are shown for C57BL/6 mice in gray and for $Slip-GC$-deficient mice in black (tick marks = 10 bp). Numbers on the x axis represent AID hotspot locations as depicted in A. These data are taken from 118 unique clones representing 55,419 bp from 28 C57BL/6J mice and 99 unique clones representing 47,798 bp from 16 $Slip-GC$-deficient mice.

**FIGURE 6.** Increased G:C transitions at the non-Ig loci $Cd79b$ and $Cd83$ in SLIP-GC-deficient mice. Mutations from sorted B220$^-$, CD19$^-$, and GL-7$^-$ B cells from PPs were analyzed in 12 2–4-month-old C57BL/6J mice and in seven 2–4-month-old SLIP-GC-deficient mice. Almost all G:C mutations from SLIP-GC-deficient mice were transitions in $Cd79b$ and $Cd83$. p values are by Fisher’s exact test.
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Given the role of AID in all aspects of immunoglobulin diversification in peripheral B cells, SLIP-GC may be an important contributor to genome stability in B cells.

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