Speckled-like Pattern in the Germinal Center (SLIP-GC), a Nuclear GTPase Expressed in Activation-induced Deaminase-expressing Lymphomas and Germinal Center B Cells*§

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We identified a novel GTPase, SLIP-GC, with expression limited to a few tissues, in particular germinal center B cells. It lacks homology to any known proteins, indicating that it may belong to a novel family of GTPases. SLIP-GC is expressed in germinal center B cells and in lymphomas derived from germinal center B cells such as large diffuse B cell lymphomas. In cell lines, SLIP-GC is expressed in lymphomas that express activation-induced deaminase (AID) and that likely undergo somatic hypermutation. SLIP-GC is a nuclear protein, and it localizes to replication factories. Reduction of SLIP-GC levels in the Burkitt lymphoma cell line Raji and in non-Hodgkin lymphoma cell lines resulted in an increase in DNA breaks and apoptosis that was AID-dependent, as simultaneous reduction of AID abrogated the deleterious effects of SLIP-GC reduction. These results strongly suggest that SLIP-GC is a replication-related protein in germinal center B cells whose reduction is toxic to cells through an AID-dependent mechanism.

The germinal center (GC)³ is a transient structure formed during T-dependent B cell responses wherein B cell affinity maturation to a specific antigen occurs, leading to the formation of high affinity memory B cells (1–3). Many features of this reaction are unique in biology such as the somatic hypermutation (SHM) of immunoglobulin (Ig) genes, the genetic rearrangement of the constant domains in class switch recombination to generate B cells bearing receptors of downstream isotypes such as IgG, IgE, and IgA, and the cellular selection process that recruits high affinity variants generated via SHM. In SHM the variable (V) regions of the heavy and light chain loci of Ig genes undergo a directed process of hypermutation where base substitutions accumulate, particularly in regions encoding the antigen binding pockets of the B cell receptor. The molecular basis for SHM is not fully understood, but it is known to be triggered by a cytosine deaminase, AID (4, 5). However, it is clear that novel factors are yet to be discovered in SHM. For example, AID alone is not sufficient for proper targeting to the Ig locus, and it is likely that a novel factor targets AID to the Ig locus (6). In addition, AID-mediated deamination of cytosines explains only mutations at G:C base pairs, yet mutations at A:T base pairs occur at approximately the same rate as G:C mutations. Although A:T mutations have been linked to the activities of the mismatch repair (MMR) proteins MSH/MSH6 and the error-prone DNA polymerase η, hypermutating Burkitt lymphoma cell lines have intact MMR and polymerase η, yet mutations at A:T base pairs are markedly reduced (7). The class switch recombination reaction is also only partly understood. Targeting of AID, the DNA substrate subjected to AID deamination, and the subsequent DNA breaks and their repair also remain only partially defined for class switch recombination. Finally, it remains unclear how these reactions are coordinated in the GC environment with both cellular selection for increased affinity to foreign antigen and tolerance mechanisms to prevent or minimize autoreactivity acquired during hypermutation that can lead to high affinity pathogenic IgG antibodies (8, 9). Clearly, efforts to understand these mechanisms and to identify novel proteins that contribute to this unique environment are needed.

To identify proteins that may contribute to SHM or other aspects of the GC reaction, we mined expression libraries generated by the I.M.A.G.E. Consortium (10) through informatics tools in the Cancer Genome Anatomy Group website (11). Given that BCL6 is a critical protein for the GC reaction (12, 13), we pooled libraries derived from GC B cells with BCL6 expression and compared them to all other libraries (see Fig. 1A for the scheme). This strategy led us to the discovery of a novel protein, SLIP-GC (speckled-like pattern in the germinal center), expressed in GC B cells, and its expression profile was...
similar to that of AID. Subsequent experiments showed that this protein is expressed in GC B cells and localizes to replication factories in the nucleus and when reduced in AID \textsuperscript{−/−} lymphoma cell lines results in an increase in DNA breaks and in cell death. These studies reveal SLIP-GC to be a novel factor that likely contributes to the unique reactions in GCs. The data also suggest that SLIP-GC reduction is toxic to B cells through an AID-mediated mechanism.

**EXPERIMENTAL PROCEDURES**

*Informatics*—To search for novel proteins expressed in GC B cells, we generated a list of B cell germinal center libraries expressing BCL6 and AID using the tBLASTn tool in GenBank\textsuperscript{TM} (14). Libraries that originated from GC B cells were then pooled (NIH_MGC_50, NCI Cancer Genome Anatomy Group GCBI, NIH_MGC_38). Using the cDNA Digital Gene Expression Displayer tool in Cancer Genome Anatomy Group, we compared gene expression of our pooled libraries against all others derived from normal tissues. Pool A, our pool, contained 64,416 sequences from 3 libraries, and pool B contained 2,924,676 sequences from 375 libraries. We then selected genes that were expressed in at least two of the three BCL6- and AID-expressing GC libraries but were rarely found in other libraries and examined expression in mouse tissues using the Unigene (15) database for correspondence. Only those genes with relatively restricted expression to peripheral lymphoid tissues in both mice and humans were considered. This analysis led to the identification of a novel protein, SLIP-GC.

**PCR of SLIP-GC in cDNA Tissue Panels**—The following cDNA panels were obtained from Clontech (BD Biosciences): human multiple tissue cDNA panel catalog no. 636742 and human immune system multiple tissue cDNA panel catalog no. 636748. SLIP-GC cDNA from lymphoid and non-lymphoid tissues was amplified (474-bp fragment) by primer pairs of human GCSP-exp-newF and human GCSP-exp-newR, 5 human immune system multiple tissue cDNA panel catalog no. 636742 and cDNA panels were obtained from Clontech (BD Biosciences):

**Northern Blot Analysis**—A probe was generated by amplifying a region near the 5' end of SLIP-GC (~300 bp) with primers pairs 5'-GCC TCG CAG TAC CTT CTA ACG GAT TGG GCC ATG AGG ACC AC-3' that amplifies the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragment (983 bp) was used as the control. 10 μl of amplified PCR product was run on a 1% agarose gel. The bands were quantitated using the Chemi-Imager-5500 gel plotting analysis program (Alpha Innotech Corp., San Leandro, CA).

**Immunoprecipitation**—For the immunoprecipitation of endogenous SLIP-GC protein, 1 x 10\textsuperscript{7} cells of B cell lymphoblast cell line CRL-2289 and CRL-2631 were incubated in a middle-size flask unstimulated as well as stimulated overnight with lipopolysaccharide. After 18 h of stimulation, the cells were lysed in RTase lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 10% glycerol, 2 mM MgCl\textsubscript{2}, 10 mM Tris-HCl, pH 7.4, 2 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 2 mM Na\textsubscript{2}VO\textsubscript{4}, and a protease inhibitor mixture (Roche Applied Science) for 10 min on ice and centrifuged for 15 min at 13,200 rpm at 4 °C. After a pre-clearing step with 1 μg of the secondary antibody and 50 μl of Protein A/G PLUS-agarose beads (Santa Cruz Biotechnolog, Santa Cruz, CA), cell extracts were incubated for 1 h at 4 °C with a C-terminal anti-SLIP-GC antibody (the polyclonal antibody was raised against the GDLGKELADVSEYKE peptide) and then incubated with an additional 50 μl of Protein A/G PLUS-agarose beads for 1 h at 4 °C. Beads were washed 4 times with 1.5 ml of lysis buffer. For Coomassie staining, beads were boiled for 5 min in 2× SDS sample buffer (125 mM Tris-HCl, 20% glycerol, 140 mM SDS, 0.3 mM bromphenol blue, 2% mercaptoethanol), fractionated by SDS-PAGE, and analyzed by Coomassie staining.

**GTPase Assay**—The GTPase activity of endogenous SLIP-GC protein was measured using the Colorimetric GTPase assay kit (high sensitivity) according to the manufacturer’s instructions (Innova Biosciences, Cambridge, UK). Briefly, after the immunoprecipitation of endogenous SLIP-GC protein described above, prewashed Protein A/G PLUS-agarose beads were resuspended in 200 μl of substrate/buffer mix and divided into wells followed by the addition of 50 μl of Gold mix per well to stop the reaction. After 2 min of room temperature incubation, 20 μl of stabilizer solution was added, and the reaction was incubated for 30 min at room temperature. After 30 min, the absorbance was recorded at 650 nm using a Benchmark microplate reader (Bio-Rad). The calculation of enzyme activity was determined with the formula (A/C)/500B (A = concentration of Pi (μM) determined from the standard curve; B = assay time in min; C = reciprocal of the enzyme dilution factor). For SDS-PAGE analysis, precipitated protein were dissociated from A/G PLUS-agarose beads by boiling for 5 min in 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.05% bromphenol blue, 2% β-mercaptoethanol), fractionated by SDS-PAGE, and analyzed by Coomassie Blue staining.

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with 106 cpm/ml 32P-labeled RNA probe. Membranes were washed twice with low stringency wash solution (Ambion) at room temperature and washed twice for 15 min at 68 °C with high stringency wash solution (Ambion). Signal was detected by exposing the membranes to phosphorimaging screens that were scanned in the Typhoon 9400 (Amer sham Biosciences) PhosphorImager. The same membranes were rehybridized with GAPDH as an internal quantitative control.

Western Blot—Proteins were separated by SDS-PAGE electrophoresis and electrotransferred to 0.45-μm Hybond ECL nitrocellulose membranes (Amer sham Biosciences) using the wet Trans-blot method in transfer buffer (0.025 M Tris, 0.192 M glycine, 2.6 mM SDS, and 20% (v/v) methanol, pH 8.3) at 100 V for 1 h. Blots were blocked overnight at 4 °C with blocking buffer (physiologic buffered saline (PBS)) with 0.1% Tween 20 and 5% fat-free milk powder (Carnation, Glendale, CA). After rinsing three times for 5 min each in PBS containing 0.1% Tween 20, blots were incubated for 1 h at room temperature with a 1:1000 dilution of N terminal SLIP-GC antibody (polyclonal raised against the GQEPHPVEDDLYKE peptide found near the N terminus of SLIP-GC within first 25 amino acids). After rinsing the membranes as above, blots were incubated for 1 h at room temperature with anti-rabbit IgG/horseradish peroxidase antibody (Amer sham Biosciences) diluted 1:2000 in blocking buffer as the secondary antibody. Immunoblots were rinsed as above and detected via an enhanced chemiluminescence method (ECL Western blotting detection system; Amer sham Biosciences).

Plasmids and Short Hairpin RNA (shRNA)—B lymphocyte cell-lines RAJI (Burkitt lymphoma), CRL-2289 (large B cell lymphoma), CRL-2630 (non-Hodgkin B cell lymphoma), and CRL-2631 (diffuse large B cell lymphoma (American Type Culture Collection (ATCC, Rockville, MD) (17–20) were used in the shRNA studies. Cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 100 μg/ml penicillin G, 100 μg/ml streptomycin, 2 mM l-glutamine, and 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) at 37 °C and 5% CO2 in a humidified incubator.

The SureSilencing shRNA plasmids for human SLIP-GC (KH18086G) and human AID (KH12741G) were obtained from SuperArray (Frederick, MD). We tested the efficacy of each plasmid for suppressing SLIP-GC and AID mRNA levels in human RAJI cells and chose the following shRNA sequences: for SLIP-GC suppression, 5'-TGG CAG ATG TGG CAG GTG AAT-3'; for AID suppression, 5'-TCTCCGcatcatgaccttccaaaggtcTTCCGTGTCaatctttgaaggtcatgatggCT-3'. The control shRNA sequence has no target genes in mammalian cells and was provided by the manufacturer: TCTCCGaatctttgaaggtcatgatggCT-3'. The control shRNA sequence has no target genes in mammalian cells and was provided by the manufacturer: TCTCCGaatctttgaaggtcatgatggCT-3'. The control shRNA sequence has no target genes in mammalian cells and was provided by the manufacturer: TCTCCGaatctttgaaggtcatgatggCT-3'.

Transient Cell Transfection—Cells were transfected using the Amaza Nucleofector® Technology (Amaza, Cologne, Germany) and Cell Line-specific Nucleofector Kit V. B cells were pre-cultured at a density of 107 cells 1 day before transfection (30-ml volume). Logarithmically growing cells were transfected using the Nucleofector programs M-013 (RAJI) or X-001 (CRL-2289, CRL-2630, CRL-2631) pulsing parameter with a final concentration of 10 μg of vector DNA followed by incubation for 30 min at 37 °C and transferred into flasks containing 14 ml of 37 °C prewarmed culture medium. After transfection, cells were cultured for 24–48 h, washed with ice-cold PBS to remove the culture medium, resuspended in cold RPMI 1640 medium, and analyzed using the BD Biosciences FACSVantage SE Flow Cytometer.

Real-time PCR (TaqMan)—Total RNA was extracted from B cells, control shRNA-transfected, and SLIP-GC-shRNA-transfected cells using the Trizol reagent (Invitrogen) according to the manufacturer’s instructions. cDNA (20 μl) was synthesized with 1 μg of total RNA as template using the SuperScript III First-Strand Synthesis SuperMix for quantitative real-time-PCR (Invitrogen) according to the manufacturer’s instructions.

Expression levels of SLIP-GC and AID were estimated using quantitative real-time PCR. The reaction was performed with the TaqMan Universal PCR Master Mix, the TaqMan MGB probes (SLIP-GC, assay ID Hs00420505_m1, and AID, assay ID Hs00210688_m1), and 6-carboxyfluorescein dye-labeled (all obtained from Applied Biosystems, Foster City, CA). A 25-μl reaction mixture containing 40 ng of total cDNA, 100 nm probe, and 200 nm primers was prepared. The PCR was run in a sealed 96-well optical plate using a qRTM5 Multicolor Real-Time PCR detection system, and the thermal cycler conditions were as follows: initial denaturation at 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of a denaturation step at 95 °C for 15 s and a primer annealing/extension step at 60 °C for 1 min. For each sample the PCR was performed in duplicate according to the manufacturer’s instructions. Melting curves of the amplified products were performed to identify the amplicon. The human actin gene served as internal control. The TaqMan assays were analyzed with the iQ5 Optical System Software, Version 2.0. For primary B cells from TLR7 mice, RNA used for real time PCR was obtained from sorted follicular B cells CD43−CD19−B220 splenic B cells. B cells were isolated from splenocytes using biotin anti-CD43 and biotin anti-CD9 antibody (BD Biosciences), and anti-biotin beads (Miltenyi Biotec, Bergisch Gladbach, Germany) were used to capture using the Miltenyi Biotec Automated Magnetic Cell Sorting machine.

In Situ Hybridization—SLIP-GC probes were generated from a 406-bp cDNA fragment subcloned into pT7T3-Pac vector. The resulting SLIP-GC vector was linearized by digestion with either EcoRI or NotI restriction enzymes. The linearized template generated from EcoRI or NotI digest was used to generate digoxigenin-labeled sense and antisense probes by invitro transcription using T3 and T7polymerase (Roche Applied Science catalog nos. 1175084001 and 11031171001), respectively. Frozen tissue sections were prepared from OCT (optimal cut-temperature)–embedded spleens and post-fixed in 4% paraformaldehyde for 10 min at 4 °C, washed in PBS at room temperature, and acetylated. RNA probes were generated from the mouse IMAGE cDNA clone EMM1002-3872135 (Open Biosystems, Huntsville, AL), added to the hybridization buffer, hybridized to tissue sections overnight at 55 °C in a humidity chamber, and washed with 5×, 2×, and 0.2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) buffer. The tissue was blocked with sheep serum, digoxigenin washing buffer, and blocking reagent (Roche Applied Science catalog no. 11585762001, Sigma-S3772). The hybridized probe was
detected after overnight incubation at 4 °C using an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Applied Science catalog no. 11093274910). Localization of SLIP-GC RNA was visualized with a nitro blue tetrazolium chloride, 5-bromo-4-chloro-3-indolyl phosphate) chromogen reaction (catalog no. 11383213001 and 11383221001, Roche Applied Science).

Flow Cytometry Analysis—SureSilencing shRNA plasmid transfection efficiency in B lymphoma cells was determined by fluorescent-activated cell sorter (FACS) by measuring GFP + cells analysis at 24 and 48 h after transfection. Cells were harvested at different times, washed with ice-cold PBS to remove the culture medium, resuspended in cold RPMI 1640 medium, and analyzed using the BD Biosciences FACSVantage SE Flow Cytometer.

Confocal Microscopy—Full-length of the coding region of the human SLIP-GC cDNA was amplified using the primer pairs 5'-CCC AAG CTT GGG ATG GCA GAA ACG AAG GAT GTT TTT GGC CAG-3' and 5'-ATA GGT ACC TTA CAG TGA TGT CCC GGG GCC AGC TCT GGT-3' and cloned into pEGFP-C3 vector (BD Bioscience) between the HindIII and KpnI sites, now called SLIP-GC-pGFP-C3, encoding a fusion of SLIP-GC with green fluorescent protein (GFP) in the C terminus.

293 cells were grown on coverslips and transfected with the SLIP-GC-pEGFP-C3 construct by FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions, and cells were incubated for 48–72 h for expression of recombinant protein. Ramos B cells were transfected by electroporation using the Gene Pulser Xcell Electroporation System (Bio-Rad) at 250 V/960 microfarad settings. After electroporation, cells were allowed to recover for 48 h and mounted on glass slides by Cytospin 4 (Thermo Electron Corp., PA). Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS for 5 min. After washing twice with PBS, cells were blocked for 1 h at room temperature with 10% normal goat serum in PBS (SC-35 and Colin) or with 4% IgG-free BSA in PBS (PCNA). Monoclonal antibodies against Colin and Splicing Factor SC-35 were obtained from Sigma and labeled with Zenon Mouse IgG labeling kit Alexa Fluor 568 (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. Labeled antibodies were diluted in PBS containing 10% normal goat serum, applied to the fixed cell, and incubated for 1 h at room temperature. Cells were fixed again with 4% formaldehyde for 15 min at room temperature, washed with PBS, and stained with DAPI (5 µg/ml) for 20 min at room temperature. For PCNA, cells were incubated with PCNA (PC10) antibody and mouse monoclonal IgG2a (Santa Cruz Biotechnology) at 1 µg/ml in 1% BSA in PBS for 1 h at room temperature. Cells were washed 3 times with 1% BSA, PBS and incubated with Zenon Alexa Fluor 568 mouse IgG2a secondary antibody (Molecular Probes) diluted to 1:5000 in 1% BSA, PBS for 1 h at room temperature in the dark. Cells were washed with PBS and stained with DAPI (5 µg/ml) for 20 min at room temperature. For all antibodies, after washing with PBS, slides were air-dried and mounted with coverslips using the Prolong Antifade kit (Molecular Probes).

Transfected cells were examined for GFP expression as well as with fluorescent-conjugated antibodies against the various proteins described above using the Zeiss LSM 510 NLO META, a laser-scanning confocal microscope. All transfections and treatments were done at least twice.

BrdUrd Staining—293 cells grown on coverslips (2 chamber slides, Nalge Nunc International Corp, Naperville, IL) were incubated in medium with 100 µM BrdUrd for 30 min at room temperature. Cells were washed and fixed with 4.0% formaldehyde in PBS and permeabilized with 0.5% Triton X-100 for 5 min followed by 3 washes with PBS. Nonspecific binding of antibodies was prevented by blocking in 0.2% fish skin gelatin for 1 h. The cells were treated with DNase I (20 units/ml) for 1 h in a buffer containing 0.2% fish skin gelatin, 0.5 mM 2-mercaptoethanol, 0.33 mM MgCl2, and 33 mM Tris-Cl, pH 8.1, at 37 °C. The DNA in the chromatin was partially digested by DNase I exposing the incorporated BrdUrd. To study BrdUrd and SLIP-GC in synchronized cells, cells were treated with 4 µg/ml aphidicolin for 24 h and then pulsed-labeled with BrdUrd for 0.5, 1, and 2 h. The cells were incubated with Anti BrdUrd Alexa Fluor 594 (1 µg/ml) for various time points, washed 3 times with PBS, and fixed again with 4% formaldehyde. The DNA was counterstained with DAPI, and slides were mounted in prolonged antifade media (Molecular Probes), dried, and viewed under confocal microscope (LSM 510 mounted on an Axiovert 200 m microscope, Carl Zeiss, Inc.).

γH2AX Staining—293 cells were grown on coverslips and transfected with the SLIP-GC-pEGFP-C3 vector as described above. Cells were treated with 10-gray γ radiation in a Cs-137 irradiator. After irradiation, cells were allowed to recover for 30 min, 1 h, and 2 h at room temperature. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS for 5 min. After washing twice with PBS, cells were blocked for 15 min at room temperature with Superblock buffer (Pierce). Cells were incubated for 1.5 h at room temperature with H2AX primary antibody (Upstate Biotechnology) diluted 1:500 in Superblock buffer. After five washes with PBS, cells were incubated for 45 min at room temperature with secondary antibody (anti-mouse Alexa Fluor 594 (Molecular Probes) diluted 1:500 in Superblock buffer. Cells were washed with PBS and stained with DAPI (5 µg/ml) for 20 min at room temperature. After washing with PBS, slides were air-dried and mounted with coverslips using the Prolong Antifade kit (Molecular Probes).

To examine DNA breaks in RAJ1 cells in response to SLIP-GC down-regulation, live, shRNA-transfected cells (which are GFP +) were sorted by FACS and washed once with ice-cold PBS, and 350,000 cells per transfection were mounted on a slide. After 5 min of air-drying, cells were fixed with 3.8% paraformaldehyde for 30 min at room temperature and washed twice with PBS. Slides were blocked with 1% BSA for 5 min at room temperature, and cells were permeabilized for 2 min on ice in 0.1% sodium citrate and 0.1% Triton X-100. Cells were washed twice with PBS and incubated overnight with H2AX and secondary antibody as described above.

Annexin V Staining—To examine apoptosis of CRL-2289 cells in response of SLIP-GC down-regulation, cells transfected with shRNA plasmids were washed, resuspended in annexin
SLIP-GC Function in Germinal Center B Cells

A. tBLASTn determination of Bcl6 AID+ GC libraries
determination of the method used to identify novel proteins primarily expressed in germinal center B cells by mining EST libraries. B. shown is the amino acid sequence of SLIP-GC. Sticky boldface motifs are potential nuclear localization signals. The underlined boldface motif is a GTPase motif (P-loop), whereas the underlined italic sequence near the C terminus is the coiled-coil region. C. shown is a representative graph of GTPase assay of immunoprecipitated SLIP-GC from lipopolysaccharide-activated and unstimulated B cells. CRL-2289 is a cell line with endogenous SLIP-GC. As a positive control, RhoA, a ubiquitious GTPase, was immunoprecipitated with specific antibodies and tested the same way as SLIP-GC. We also performed GTPase assays on CRL-2631, which does not express SLIP-GC. Accordingly, a Coomassie Blue gel of immunoprecipitated SLIP-GC only shows a SLIP-GC band in the CRL-2289 extracts (data not shown), and SLIP-GC GTPase activity in lipopolysaccharide (LPS)-activated CRL-2631 was negligible, whereas RhoA GTPase activity was high (SLIP-GC, 0.36 nmol GDP/min; RhoA, 28.48 nmol GDP/min). The assay was done at least two times.

Terminal dUTP Nick-end Labeling Assay—In situ nick end-labeling of nuclear DNA fragmentation was performed with the In situ Cell Death Detection Kit (Roche Applied Science). Briefly, after the FACS, GFP+ cells were washed once with ice-cold PBS, and 350,000 cells per transfection were mounted on a slide (Sigma). After air drying, cells were fixed with 3.8% paraformaldehyde for 30 min, washed twice with PBS, covered, and analyzed by confocal microscopy. The number of apoptotic cells was determined by counting all cells per view until reaching ~100 cells per sample using a confocal microscope.

Statistics—All comparisons were carried out using the non-parametric Mann-Whitney Wilcoxon test, and p values equal to or less than 0.05 were considered significant.

RESULTS

Expression analysis of GC B cell libraries from the I.M.A.G.E. consortium led us to the identification of SLIP-GC, a novel GTPase, annotated in GenBank™ accession number Q6BC16 as an open reading frame corresponding to a hypothetical protein. Using the protein sequence to run a series of psi-blast iterations (16) revealed no similarity over threshold to any known proteins, although hypothetical proteins with significant homology were found among vertebrates, and distant homologs were found among a variety of invertebrates. Among them was a mouse homolog with very high similarity (~80%) whose expression profile was strikingly similar to human SLIP-GC (accession number XM_983339; data not shown). The iterations picked up mostly GTPases, such as dynamin-related, GTP1-OBG, and interferon-γ-induced GTPase, all having less than 20% homology and below threshold. This result was driven by a highly conserved ATP/GTP-binding site motif A (P-loop) domain near the N terminus (Fig. 1B), a domain that was detected using Prosite (17) and InterProScan (18). No other putative domains were found except several nuclear localization signals throughout the protein and a coiled-coil region in the C terminus detected with the PSORT II program (19). GTPase assays confirmed that SLIP-GC has GTPase activity, which was greatly enhanced upon stimulation with lipopolysaccharide (LPS, Fig. 1C). Combined, these results suggest that SLIP-GC is a nuclear protein belonging to a novel family of GTPases.

SLIP-GC Is Expressed in GC B Cells and in Lymphoma Cell Lines with Active SHM—To confirm our “virtual” results, we examined expression by reverse transcription-PCR in a cDNA panel derived from various human tissues including lymphoid tissues of interest, such as lymph nodes, spleen, and tonsil. The expression pattern of SLIP-GC in human tissues was similar to that seen in the EST libraries, except for additional expression in the liver and to a lesser extent kidney (Fig. 2A). No other putative domains were found except several nuclear localization signals throughout the protein and a coiled-coil region in the C terminus detected with the PSORT II program (19). GTPase assays confirmed that SLIP-GC has GTPase activity, which was greatly enhanced upon stimulation with lipopolysaccharide (LPS, Fig. 1C). Combined, these results suggest that SLIP-GC is a nuclear protein belonging to a novel family of GTPases.

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SLIP-GC Function in Germinal Center B Cells

To examine expression of SLIP-GC in B cell lymphoma patients, real time PCR of both SLIP-GC and AID was used with a panel using normal lymphoid tissues, Hodgkin lymphomas, or large diffuse B cell lymphomas. These results strongly suggest that SLIP-GC is expressed in GC B cells.

To further define the cells with SLIP-GC expression within peripheral lymphoid tissues, we examined SLIP-GC levels in follicular splenic B cells (CD43+/CD95+ B220+) from TLR7 transgenic mice that spontaneaously form numerous large GCs.4

The expression of SLIP-GC in germinal center B cells was compared with that of several proteins involved in the germinal center reaction (BCL6, c-Myc, and AID) and/or terminal differentiation into antibody-secreting switched B cells (Blimp-1 and AID). SLIP-GC expression was more similar to that of c-Myc than AID (supplemental Fig. 2A). However, the in vitro activation assay described above activates class switch recombination but not SHM. Therefore, to assess if signals inducing immunoglobulin hypermutation also induce SLIP-GC expression, the immunoglobulin receptors of BL2 cells (a lymphoma cell line) were crossed-linked simultaneously with treatment with anti-CD19 and anti-CD21, a mixture that induces hypermutation in these cells without further increasing AID transcript levels (24). This resulted in increased SLIP-GC levels but unaltered AID transcript levels (supplemental Fig. 2B).

Finally, to determine in vivo SLIP-GC expression, in situ hybridization with a SLIP-GC antisense probe was done in serially cut spleens stained with PNA (a marker for germinal center B cell clusters within the follicles of peripheral lymphoid tissues) after immunization with the NP hapten (4-hydroxy-3-nitrophenyl acetyl-hapten). SLIP-GC transcripts were seen in cells that co-localized with PNA+ cells forming the typical GC B cell clusters (Fig. 3). Similarly, SLIP-GC was expressed in GL7+ B cells of immunized AID-proficient and AID-deficient mice (supplemental Fig. 2C), suggesting that AID expression does not influence SLIP-GC expression in GC cells. Because activated peripheral CD8+ T cells can also bind PNA, we performed real time PCR using sorted CD8+ T cells to compare with CD19+ A+ germinal center B cells from the same spleens after immunization with NP, a reaction that enriches for A+ B cells undergoing affinity maturation. The B cells had a 10–27-fold increase in the levels of SLIP-GC compared with the T cells that had negligible levels of SLIP-GC (supplemental Fig. 2D). Combined, these results strongly suggest that SLIP-GC is expressed in GC B cells.

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To examine expression of SLIP-GC in B cell lymphoma patients, real time PCR of both SLIP-GC and AID was used with a panel using normal lymphoid tissues, Hodgkin lymphomas, or large diffuse B cell lymphomas, the last having being correlated with dysregulation of AID activity and with having its cellular origins with GC B cells (25, 26). SLIP-GC and AID levels were highest in patients with large diffuse B cell lymphomas (Fig. 4; Mann-Whitney p < 0.05 when comparing SLIP-GC or AID in large diffuse B cell lymphomas to normal or to Hodgkin lymphomas). These results demonstrate what we observed in lymphoma cell lines; although not necessarily at the same levels,

4 S. Bolland and P. Pisitkun, manuscript in preparation.
SLIP–GC and AID are expressed in the same B cells. Indeed, the combined expression studies with hypermutating lymphoma cell lines, lymphoma patient samples, primary B cells induced to express AID in vitro and follicular or, specifically, germinal center B cells from mice demonstrate that SLIP–GC is expressed in GC B cells likely undergoing SHM.

**FIGURE 5.** SLIP–GC Localizes to Replication Factories—To gain insight into its function, we examined SLIP–GC intracellular localization by confocal microscopy in fibroblasts (HEK293) and in B cells (Ramos cell line) by transiently transfecting cells with a vector encoding a fused complex of SLIP–GC and GFP. SLIP–GC localized to the nucleus and formed a speckled pattern (Fig. 5A) reminiscent of nuclear proteins involved in DNA transactions such as replication, repair, and transcription as well as RNA splicing proteins and certain structural proteins found in Cajal bodies. Therefore, to determine whether the SLIP–GC speckled pattern is associated with any of these mechanisms, we examined co-localization with 1) the DNA replication processivity factor PCNA (27), 2) the RNA splicing factor, SC35 (28), and 3) the nuclear structural protein typically associated with Cajal bodies, coilin (29). In addition, to examine if SLIP–GC associates with the transcription machinery, transfected cells were treated with a transcription inhibitor (α-amanitin), reasoning if SLIP–GC is associated with transcription, its speckle pattern would be disturbed with inhibition of transcription. Finally, to determine whether SLIP–GC associates with the DNA double-strand break repair machinery and/or with replication factories, we examined co-localization of SLIP–GC with H2AX foci after exposure to ionizing radiation and with replication factories after aphidicolin-mediated replication arrest and release and a BrdUrd pulse.

SLIP–GC did not significantly co-localize with SC35, with coilin, or to sites of H2AX phosphorylation after ionizing radiation treatment (Fig. 5B) nor was the speckle pattern affected by treatment with α-amanitin (data not shown). On the other hand, SLIP–GC speckles overlapped with PCNA, and after aphidicolin arrest and release, the great majority of the SLIP–GC speckles coloculated with BrdUrd-stained replication factories (Fig. 5B). Indeed, we noticed that replication factory distribution throughout the cell cycle was strikingly similar to the variation seen in SLIP–GC speckles in some cells (Fig. 5C). The overlap between SLIP–GC speckles and BrdUrd-stained replication factories was reflected in both HEK293 cells overexpressing the protein and in Ramos B cells (Fig. 5D). Taken together, these data strongly suggest that SLIP–GC localizes to replication factories.

SLIP–GC Reduction in Lymphoma Cell Lines Is Toxic to Cells, but the Effect Is Reversed with Simultaneous Reduction in AID Levels—We examined expression of SLIP–GC and AID in an array of lymphoma cell lines (non-Hodgkin and Burkitt) and found that both proteins are expressed in the same cell lines, albeit not always at the same level (Fig. 6, A and B;
impact of SLIP-GC reduction was observed with two independent shRNA sequences. As expected, this treatment did not impact CRL-2630 or CRL-2631 cells where SLIP-GC is expressed at minimal levels or not at all (Fig. 6, A and E). Therefore, it appears that increasing SLIP-GC levels triggers autoregulatory mechanisms to maintain a maximum level, but decreasing the levels even modestly reduces the recovery of live cells, perhaps by increased apoptosis or decreased proliferation.

Although reducing AID levels alone had no impact on any of the cell lines examined, strikingly, simultaneous reduction of SLIP-GC and AID not only reversed the phenotype resulting from SLIP-GC reduction but improved live cell recovery over cells transfected with scrambled by 3-fold in Raji cells and by 50% in CRL-2289, a cell line with naturally higher levels of SLIP-GC (Fig. 6E). For the double transfectants we used a neomycin vector for one of the proteins instead of GFP to prevent doubling the amount of GFP-positive cells simply from increased amounts of the GFP vector. Indeed, double transfectants of SLIP-GC or AID with scramble shRNA did not result in a similar increase (data not shown). Double-transfected cells lacking endogenous SLIP-GC and AID were unaltered by the treatment (CRL-2630 and CRL-2631, Fig. 6E). Interestingly, SLIP-GC reduction did not appear to impact proliferation in these cells (supplemental Fig. 3). Taken together, these data suggest that SLIP-GC levels are important in AID+ B cells because the protein provides some kind of protection against AID activity or at least from levels of AID protein.

shRNA against SLIP-GC Causes Apoptosis and an Increase in DNA Double-strand Breaks—Given the reversal of the phenotype in SLIP-GC shRNA-treated cells with simultaneous reduction of AID and SLIP-GC localization to replication factories, it is possible that SLIP-GC protects replicating DNA against AID-mediated double strand breaks. Therefore, we measured the number of live sorted transfected B cells with phosphorylated H2AX foci. Single transfectants of shRNA-SLIP-GC experienced a more than 3-fold increase in the number of cells with detectable double-strand breaks, and again, this effect was reversed by simultaneous reduction in AID levels (Fig. 7A). As described in a previous study (30), late-stage apoptotic cells stained positive for H2AX but were not considered in the analysis (Fig. 7B).

supplemental Fig. 1). Several attempts to generate stable transfectants overexpressing SLIP-GC in B cells failed to increase protein levels due to a reduction in expression of endogenous transcripts; whereas transcript levels remained essentially unchanged, sequencing of the band from transfectants revealed that most were generated from the vector and not from the endogenous gene (data not shown). This suggested that, unlike forced expression in HEK293 cells where SLIP-GC is not normally expressed and where the cells appeared unaffected, the levels of SLIP-GC in B cells, where it is naturally present, are tightly regulated.

Next, we examined the impact of reducing SLIP-GC levels using RNA interference vectors with simultaneous expression of an shRNA against a target transcript and a marker such as neomycin resistance or GFP. This strategy lowered expression of SLIP-GC transcripts by at least 50% in Raji cells (Fig. 6C). Similarly, an shRNA against AID lowered AID levels by 70% (Fig. 6D). In SLIP-GC-deficient cells, there was a reduction in the fraction of live GFP+ cells recovered via flow cytometry of about 50% in Raji and 30% in CRL-2289, a cell line with high levels of endogenous SLIP-GC (Fig. 6E). Reducing AID levels did not impact the levels of live transfected cells (Fig. 6E). The
Given that DNA breaks can induce apoptosis, live GFP<sup>+</sup> cells were sorted at 48 h after transfection and stained with annexin V for early stage apoptosis (before DNA fragmentation) (Fig. 8A) or examined with the terminal dUTP nick-end labeling assay (TUNEL) for late-stage apoptosis (Fig. 8B). shRNA-SLIP-GC single transfectants had a significant increase in the number of apoptotic cells compared with scramble-transfected, and this was observed regardless of the method used. This phenotype was reversed by simultaneous reduction of AID with shRNA-AID (Fig. 8A). However, given the increase in the number of apoptotic cells in SLIP-GC-deficient cells, it is possible that some of the observed cells with DNA breaks were in the initial stages of DNA fragmentation, making it difficult to definitively conclude that the observed DNA breaks are the direct result of AID rather than ongoing DNA fragmentation. However, the results point to these being AID-mediated breaks because the cells used in the analysis appeared neither overtly apoptotic nor to be undergoing chromosome condensation. In addition, H2AX staining in apoptotic cells tends to cover the entire cells, whereas only a few breaks were detected per cell among the apparently healthy cells used in the analysis (Fig. 7B). Nevertheless, although it is formally impossible at this point to definitively distinguish between these possibilities, it is clear that SLIP-GC reduction causes cell death in B cell lymphoma cells lines through an AID-dependent mechanism.

DISCUSSION

Through analysis of EST libraries expressing BCL6 and AID derived from GC B cells, we identified a novel GTPase, SLIP-GC. This protein is highly expressed in GC B cells and their derived lymphomas as evidenced by in situ hybridization and by expression analysis in AID<sup>+</sup> B cell lymphoma cell lines and in patient samples with large diffuse B cell lymphoma. It localizes to replication factories, and its levels appear to be tightly regulated but only in B cells; overexpression of this protein in non-lymphoid cells was of no detectable consequence, whereas in B cells it resulted in a decreased expression of the endogenous gene.

Reducing SLIP-GC in B cell lines with AID expression resulted in an increase in the number of cells with DNA double-strand breaks and in the number of apoptotic cells. However, simultaneous reduction of AID levels reversed this phenotype, suggesting that SLIP-GC acts as a negative regulator of AID activity. Given its localization to replication factories, one can imagine that SLIP-GC blocks access of AID to replicating DNA
to protect it from deamination and subsequent DNA strand break formation. Alternatively, SLIP-GC may regulate AID protein levels. For example, the increase in survival with double transfection may be explained if high AID levels make cells prone to apoptosis, and SLIP-GC curtails that effect by maintaining AID levels. Additionally, the fact that the increase in survival in the double transfectants exceeds the survival in cells with reduction of AID alone suggests that high SLIP-GC levels are also toxic. Perhaps, SLIP-GC plays an important role in coordinating origins of replication in these very rapidly dividing cells so as to prevent more than one round of replication of a given region. The highly conserved GTPase motif near the N terminus of SLIP-GC resembles that of YqeH, a GTP-binding protein whose depletion results in excess initiation of DNA replication (31). This possibility is less likely to explain why simultaneous reduction of AID reverses the phenotype in SLIP-GC-deficient cells. Nevertheless, these possibilities make specific predictions in mice lacking the gene encoding SLIP-GC; if the role of this protein is in coordination of DNA replication, the morphology of the GC should be altered, because cell division in these cells occurs at very high rates, and a disruption in replication is likely to profoundly impact the GC structure. On the other hand, if SLIP-GC is involved in protecting replicating DNA from AID, then deletion of the SLIP-GC gene in mice could result in untargeted AID activity throughout the genome. This may result in increased susceptibility of these mice to AID-dependent B cell lymphomas.

Acknowledgments—We are grateful to Jan Drake for critical reading of the manuscript and for suggestions for the potential function of SLIP-GC. Special thanks are also to Donald Cook for comments on the manuscript and to Ming Lang Zhao, Pamela Blackshear, Tony Xiao, and Chuanjiang Jiang for help with experiments.

REFERENCES
1. Berek, C., Berger, A., and Apel, M. (1991) Cell 67, 1121–1129
2. Camacho, S. A., Kosco-Vilbois, M. H., and Berek, C. (1998) Immunol. Today 19, 511–514
3. Weigert, M. G., Cesari, I. M., Yonkovich, S. J., and Cohn, M. (1970) Nature 228, 1045–1047
4. Muramatsu, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinkai, Y., and Honjo, T. (2000) Cell 102, 553–563
5. Revy, P., Muto, T., Levy, Y., Geissmann, F., Plebani, A., Sanal, O., Clark, A. B., Rogozin, I. B., and Diaz, M. (2007) Mol. Immunol. 44, 2659–2666
6. Xiao, Z., Ray, M., Jiang, C., Clark, A. B., Rangozin, I. B., and Diaz, M. (2007) J. Immunol. 178, 7422–7431
7. Jiang, C., Zhao, M. L., and Diaz, M. (2009) Immunology 126, 102–113
8. Lennon, G., Auffray, C., Polymopoulos, M., and Soares, M. B. (1996) Genomics 33, 151–152
9. Strausberg, R. L., Bueto, K. H., Emmert-Buck, M. R., and Klausner, R. D. (2000) Trends Genet. 16, 103–106
10. Ye, B. H., Cattoretti, G., Shen, Q., Zhang, J., Hawe, N., de Waard, R., Leung, C., Nouri-Shirazi, M., Orazi, A., Chaganti, R. S., Rothman, P., Stall, A. M., Pandolfi, P. P., and Dalla-Favera, R. (1997) Nat. Genet. 16, 161–170
11. Staadt, L. M., Dent, A. L., Shaffer, A. L., and Yu, X. (1999) Int. Rev. Immunol. 18, 381–403
12. Madden, T. L., Tatusov, R. L., and Zhang, J. (1996) Methods Enzymol. 266, 131–141
13. Boguski, M. S., and Schuler, G. D. (1995) Nat. Genet. 10, 369–371
14. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
15. de Castro, E., Sigrist, C. J., Gattiker, A., Bulliard, V., Langendijk-Jaekel, P. S., Gasteiger, E., Bairoch, A., and Hulo, N. (2006) Nucleic Acids Res. 34, W362–W365
16. Quevillon, E., Silvestoinen, V., Pillai, S., Harte, N., Mulder, N., Apweiler, R., and Lopez, R. (2005) Nucleic Acids Res. 33, W116–W120
17. Horton, P., and Nakai, K. (1999) Proc. Int. Conf. Intell. Syst. Mol. Biol. 5, 147–152
18. Pulvertaft, J. V. (1966) Lancet 1, 238–240
19. Beckwith, M., Longo, D. L., O’Connell, C. D., Moratz, C. M., and Urba, W. J. (1990) J. Natl. Cancer Inst. 82, 501–509
20. Benjamin, D., Magrath, I. T., Maguire, R., Janus, C., Todd, H. D., and Parsons, R. G. (1982) J. Immunol. 129, 1336–1342
21. Gabay, C., Ben-Bassat, H., Schlesinger, M., and Laskov, R. (1999) Eur. J. Haematol. 63, 180–191
22. Faili, A., Aoufouchi, S., Guérandier, Q., Zober, C., Léon, A., Bertocci, B., Weill, J. C., and Reynaud, C. A. (2002) J. Biol. Chem. 277, 7422–7431
23. Gabay, C., Ben-Bassat, H., Schlesinger, M., and Laskov, R. (1999) Eur. J. Haematol. 63, 180–191
24. Pasqualucci, L., Bhagat, G., Jankovic, M., Compagno, M., Smith, P., Muramatsu, M., Honjo, T., Morse, H. C., 3rd, Nussenzeig, M. C., and Dalla-Favera, R. (2008) Nat. Genet. 40, 108–112
25. Pasqualucci, L., Neumeister, P., Goossens, T., Janjuch, G., Chandrani, R. S., Küppers, R., and Dalla-Favera, R. (2001) Nature 412, 341–346
26. Aragno, M., and Berset, C. (2005) J. Cell. Biochem. 91, 56–67
27. Fu, X. D., and Maniatis, T. (1990) Nature 343, 437–441
28. Andrade, L. E., Chan, E. K., Raska, I., Peebles, C. L., Roos, G., and Tan, E. M. (1990) J. Natl. Cancer Inst. 82, 501–509
29. Oriani, A., Chaganti, R. S., Kuipers, R., and Dalla-Favera, R. (2008) Methods Enzymol. 412, 341–346
30. Sonananathan, S., Suchyna, T. M., Siegel, A. J., and Berezney, R. (2001) J. Biol. Chem. 276, 56–67
31. Boguski, M. S., and Schuler, G. D. (1995) Nat. Genet. 10, 369–371
32. Patton, W. M. (2000) J. Biol. Chem. 275, 9390–9395
33. Morimoto, T., Loh, P. C., Hirai, T., Asai, K., Kobayashi, K., Moriya, S., and Ogasawara, N. (2002) Microbiol. Immunol. 46, 3539–3552