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GABPβ2 Is Dispensible for Normal Lymphocyte Development but Moderately Affects B Cell Responses*

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Xuefang Jing[‡], Dong-Mei Zhao[‡], Thomas J. Waldschmidt[§], and Hai-Hui Xue[‡1]

From the Departments of [‡]Microbiology and [§]Pathology, Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242

GA-binding protein (GABP) is the only Ets family transcription factor that functions as a heterodimer. The GABPα subunit binds to DNA, and the GABPβ subunit possesses the ability to transactivate target genes. Inactivation of GABPα caused embryonic lethality and defective lymphocyte development and immune responses. There are 3 isoforms of the GABPβ subunit, but whether they have distinct functions has not been addressed. In this study, we selectively ablated the expression of GABPβ2 using a gene trap strategy. GABPβ2-deficient mice were viable and had normal T and B cell development, suggesting that loss of GABPβ2 is compensated for by other GABPβ isoforms during these processes. GABPβ2-deficient T cells can be activated and proliferate similarly to wild-type controls. In contrast, B cells lacking GABPβ2 showed 2–3-fold increases in proliferation in response to B cell receptor stimulation. In addition, GABPβ2-deficient mice exhibited moderately increased antibody production and germinal center responses when challenged with T-dependent antigens. These results indicate that albeit GABPβ isoforms are redundant in lymphocyte development, GABPβ2 has a distinct role in restraining B cell expansion and humoral responses.

GA-binding protein (GABP)[2] is a member of the *Ets* family transcription factors and is comprised of two subunits, GABPα and GABPβ (1). GABPα contains a DNA binding *Ets* domain, which is conserved among all the Ets factors and is of ~85 amino acids in length. The *Ets* domain assumes a winged helix-loop-helix configuration and binds preferentially to a purine-rich consensus DNA sequence containing GGA(A/T). On the other hand, GABPβ cannot bind DNA but contains 4 ankyrin repeats in its N terminus, which mediate the protein-protein interaction with GABPα. GABPβ also contains a nuclear localization signal, which targets the GABPα/β dimer into the nucleus. Transactivation activity of the GABPα/β complex is considered to reside in the C terminus of the GABPβ subunit, but the exact location has not been unequivocally mapped.

The GABPα/β complex has versatile roles in regulating basic cellular functions and tissue-specific functions (1–3). Gene-targeting studies of the DNA-binding GABPα subunit have revealed its critical roles during embryogenesis (4), reentry into the cell cycle (5–8), and in synaptic function at the neuromuscular junction (9, 10). In lymphocytes, GABP activates an interleukin (IL)-2 enhancer (11), IL-16, and Fas promoters (12, 13), and transcription of IL-7Rα (14, 15). Recently, we showed that GABP is a key component of a gene regulatory network programming B lineage commitment and differentiation by directly regulating Pax5 gene expression (16). In contrast to extensive studies on GABPα, the roles of the GABPβ subunit *in vivo* have not been investigated. The most studied GABPβ isoforms are those encoded by the *Gabpb1* allele, which generates two protein products, GABPβ1L (originally named GABPβ1 or GABPβ1-1) and GABPβ1S (originally named GABPβ2 or GABPβ1-2), via alternative splicing (17, 18). The N-terminal 332 amino acids of both GABPβ1L and GABPβ1S isoforms are identical, but their C termini differ in length and sequence. GABPβ1L has a longer C-terminal tail (50 amino acids), which adopts a leucine zipper-like structure, forming homodimers and even an $\alpha_2\beta_2$ GABP tetramer when two Ets motifs are adjacent or brought into proximity (17, 19, 20). In contrast, the C terminus of the shorter isoform, GABPβ1S, is 15 amino acids long, lacks the leucine zipper-like structure, and thus cannot form homodimers or tetramers. Nevertheless, both GABPβ1L and GABPβ1S heterodimerize with GABPα with similar affinity (21). It has been disputed over whether the leucine zipper-like structure in GABPβ1L has a unique role in transactivation of GABP target genes. In a recent report, we specifically targeted GABPβ1L by deleting exon 9 of the *Gabpb1* gene that encodes the entire leucine zipper-like structure without eliminating the GABPβ1S isoform (22). In contrast to a pre-implantation lethality in a GABPα-null mutant, GABPβ1L$^{-/-}$ mice were viable. On the other hand, targeting both GABPβ1L and GABPβ1S caused embryonic lethality prior to embryonic day 12.5 (22). These findings indicate that the *Gabpb1* gene products are required for normal embryogenesis, whereas loss of GABPβ1L expression can be compensated for by other GABPβ isoforms including GABPβ1S.

A third GABPβ isoform, GABPβ2 (originally named GABPβ2-1), is encoded by *Gabpb2* (18) and is 414-amino acids long with its N-terminal ankyrin repeats (amino acids 1–130) sharing 87% identity with GABPβ1 isoforms. GABPβ2 also has a long C terminus, with its 317–366 amino acid residues sharing 70% identity with that of GABPβ1L and also adopting a leucine zipper-like structure. The 367–414 amino acid residues in GABPβ2 are unique. The C terminus of GABPβ2 cannot only

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[1] To whom correspondence should be addressed: 51 Newton Rd. BSB 3–710, IA City, IA 52246. Tel.: 319-335-7937; Fax: 319-335-9006; E-mail: hai-hui-xue@uiowa.edu.

[2] The abbreviations used are: GABP, GA-binding protein; IL, interleukin; SRBC, sheep red blood cells; GC, germinal center; BCR, B cell receptor; TLR, toll-like receptor; LPS, lipopolysaccharide; CFSE, carboxy-fluorescein diacetate succinimidyl ester; ELISA, enzyme-linked immunosorbent assay; WT, wild type; DN, double negative; RACE, rapid amplification of cDNA ends; HA, hemagglutinin.

mediate the formation of GABPβ2 homodimers but also mediates the heterodimerization of GABPβ2 and GABPβ1L (18). Since its initial cloning, GABPβ2 has not been studied, and its function is completely unknown. The structural similarity raised the possibility that GABPβ isoforms may have distinct and overlapping roles in regulating lymphocyte development and functional responsiveness. As one of the first steps aiming to dissect the exact roles of each GABPβ isoform, we have inactivated the *Gabpb2* gene using a gene trap strategy.

EXPERIMENTAL PROCEDURES

5′-RACE and RT-PCR—Splenic B cells were negatively selected using EasySep B cell enrichment kits (StemCell Technology), and total RNA was extracted from purified B cells or total thymocytes using the RNeasy Mini kit (Qiagen), and 5′-RACE was done with the GeneRacer kit (Invitrogen). Racer and nest racer primers were supplied in the kit, and antisense primers complementary to mouse GABPβ2 cDNA (5′-GCAGCTTCTAGCAGCCTCTTC and 5′-TTCCCCAAGTCCACCAGAGAC) were used in RT-PCRs (Fig. 1*A*). Nested PCR was performed to increase the specificity of amplification. PCR products were subcloned into the pCR4-TOPO vector with a TOPO TA cloning kit (Invitrogen) and then sequenced.

Generation of GABPβ2-targeted Mice—An ES clone (RRJ488) was obtained from the International Gene Trap Consortium through the Mutant Mouse Regional Resource Center of the University of California at Davis. Mapping of the insertion site of the reporter gene is done using the Expand Long Template PCR System (Roche Applied Science). The ES cells were microinjected into C57BL/6 blastocysts, and male chimeras were identified to achieve germline transmission. GABPβ2$^{+/tp}$ mice were interbred to obtain GABPβ2$^{tp/tp}$ and littermate controls. All experiments with mice followed protocols approved by the Institutional Animal Care and Use Committee, University of Iowa.

Generation of GABPβ2 Antisera and Western Blotting—To generate antisera that are specific to GABPβ2 with no cross-reaction with GABPβ1, we used a recombinant GST fusion protein expressing GABPβ2 C-terminal amino acids 275–414 as an antigen, because the N termini of all GABPβ isoforms contain highly conserved ankyrin repeat domains. To this end, we PCR-amplified cDNA corresponding to the GABPβ2 C-terminal fragment in a pGEX-4T-1 vector and expressed the fusion protein in BL21 Star competent cells (Stratagene). The fusion protein was expressed at high abundance with expected molecular weight and purified with magnetic resin-based MagneGST particles (Promega). The expression of GABPβ2 was confirmed by MALDI-TOF mass spectrometry, and the purified protein was used to immunize rabbits for antibody production (Bio-synthesis). To test the specificity of the GABPβ2 antiserum, we cloned GABPβ1L and GABPβ2 cDNA in pCruz-HA vector (Santa Cruz Biotechnology) so that an HA tag is fused to the N terminus of each expressed protein. We then expressed these proteins in 293 HEK cell lines, and subjected the cell lysates to immunoblot with anti-HA antibody, which detected both GABPβ1L and GABPβ2 of expected molecular weight (Fig. 2*B*). In contrast, the GABPβ2 antiserum detected GABPβ2 at a 1:2000 dilution but did not cross-react with GABPβ1 (Fig. 2*C*). For detection of GABPβ2 expression, whole cell extracts were prepared from thymocytes, splenocytes, and splenic B cells as described previously (23). Lysate protein (30 μg) was separated on SDS/PAGE gels, transferred to nitrocellulose, and immunoblotted with the GABPβ2 antiserum (14). An anti-GABPα antibody (H180, Santa Cruz Biotechnology) was used to detect GABPα expression, and an antiserum raised against the N terminus of GABPβ1 (14) was used to detect both GABPβ1L and GABPβ1S.

Flow Cytometry and CFSE Staining—Single cell suspensions were prepared from thymuses, spleens, and bone marrow, and stained with fluorochrome-conjugated antibodies, as described (16). All fluorochrome-conjugated antibodies were from BD PharMingen or eBiosciences. Negatively selected splenic B cells were labeled with CFSE as described (16), stimulated with 10 μg/ml of anti-IgM, and dilution of CFSE was determined on different days by flow cytometry.

[^{3}H]Thymidine Uptake—Splenic T and B cells were purified by negative selection using EasySep T and B cell enrichment kits (StemCell Technology), respectively, and the purity of isolated cells was ~95%. T cells (1×10^5/well) were stimulated with plate-bound anti-CD3 (0.25 or 0.5 μg/ml, clone 145-2C11, BD Biosciences) in the absence or presence of anti-CD28 (1 μg/ml, clone 37.51, BD Biosciences). B cells (0.6×10^5/well) were stimulated with 2.5 μg/ml anti-IgM μ chain (Jackson ImmunoResearch Laboratories), 5 μg/ml anti-CD40 (BD PharMingen), 5 μg/ml LPS (Sigma), or 1 μg/ml CpG oligonucleotides (ODN1862, Invivogen). The cells were cultured in 96-well plates in triplicate for each condition for 48–60 h and pulsed with 1 μCi of [^{3}H]thymidine (PerkinElmer) for the last 12 h of culture. Radioactivity incorporated into cells was collected on a UniFilter-96 (GF/B, PerkinElmer) with a cell harvester and was counted using a TopCount.NXT microplate scintillation and a luminescence counter (Packard).

Immunization and Enzyme-linked Immunosorbent Assay (ELISA)—To determine the immune response to a T-independent antigen, mice were immunized intraperitoneally with 100 μg of TNP(52)-AECM-Ficoll (Biosearch Technologies), and the sera were collected on day 8 after immunization. For immune responses to a T-dependent antigen, mice were intraperitoneally injected with 100 μg of Imject Ovalbumin (Pierce) mixed with Imject Alum (Pierce, volume 1:1), boosted 1 week later with the same regimen, and sera collected after another week. The sera were diluted in 1:2 series to a total of 12 points, and TNP- or ovalbumin-specific immunoglobulins (Igs) were measured by ELISA. In brief, high binding plates (Immulux, Dynex) were coated with either 20 μg/ml of TNP(38)-BSA (Bioresearch Technologies) or 5 μg/ml of Imject Ovalbumin to absorb antigen-specific Igs, which were then detected with biotin-conjugated rat anti-mouse antibodies specific for murine IgM, IgG1, and IgG3. Visualization of the antigen-antibody complexes was revealed with the avidin-horseradish peroxidase and TMB substrate set (BD PharMingen), and absorbance at 450 nm was read on an ELX800 microplate reader (Bio-TEK). Linear absorbance readings were observed within one particular range of serum dilutions, and these absorbance readings

were used to compare antigen-specific Ig titers in WT and GABPβ2-deficient mice.

Sheep Red Blood Cell (SRBC) Immunization and Immunohistochemistry—Mice were *i.p.* injected with 0.2 ml of 10% v/v SRBC suspension (Colorado Serum Company) in phosphate-buffered saline, and spleens were harvested on days 8, 12, and 18 postimmunization. Splenocyte suspensions from half of the spleen were stained with anti-B220 mAb along with fluorescein isothiocyanate-conjugated peanut agglutinin (PNA, Vector Laboratories), and analyzed for GC B cell responses by flow cytometry. The other half of the spleen was used to determine GC structures by immunohistochemistry as described (24).

RESULTS

Structure of 5′-Untranslated Regions in the Gabpb2 Gene—In our previous studies, we used ES clones that have a *Gabpa* allele inactivated by a gene trap strategy to generate GABPα-deficient mice (14, 16). In this method, ES cells were retrovirally transduced with a reporter gene, such as βgeo, which is a fusion of β-galactosidase and a neomycin resistance gene. When the reporter gene is integrated into the ES genome and inserted in an intron of a gene, the gene is thus trapped. Because a strong splice acceptor is placed immediately 5′ of the reporter gene after the trapped gene is transcribed, the splicing will occur between the upstream introns and the reporter gene, resulting in truncation and/or inactivation of the trapped gene. By searching the data base of the International Gene Trap Consortium, we identified an ES clone (named RRJ488) which has an insertion of the βgeo reporter gene in the *Gabpb2* locus. The insertion presumably occurred in an intron that is downstream of a non-coding exon at the 5′-end of the locus. The information on the *Gabpb2* gene structure suggests existence of three 5′ non-coding exons and alternative usage of the exons. If this approach is to be used to study the effect of *Gabpb2* inactivation in lymphocytes obtained from RRJ488 ES clone-derived mice, it is important to determine whether these exons are utilized in B and T cells. We therefore used rapid amplification of cDNA 5′-end assay (5′-RACE) to determine the transcription initiation sites and the usage of 5′ non-coding exons in the *Gabpb2* gene. We performed nested RT-PCRs on splenic B cells (Fig. 1*A*) and observed three major transcripts (Fig. 1*B*). Analysis of the sequences of each transcript revealed that transcription of the *Gabpb2* gene can be initiated from three different sites. If we arbitrarily define a location 12,480 bp upstream of exon 2 as "+1," transcription can be initiated from +959, +1042, and +1118 bp, and that the three 5′ non-coding exons are utilized differently in each transcript (Fig. 1*C*). We also characterized *Gabpb2* transcription initiation sites in thymocytes and observed two major transcripts that correspond to Transcripts 1 and 3 in B cells (data not shown). These results indicate that although transcription of the *Gabpb2* gene can be initiated from different locations and there is different usage of 5′ non-coding exons, the protein product from these transcripts is not altered.

GABPβ2 Expression Is Completely Abolished in GABPβ2$^{tp/tp}$ Mice—We then mapped the insertion site of the βgeo reporter gene in the RRJ488 ES cells using long template PCR. Based on the sequence tags provided for the ES clone, sense primers were designed based on the genomic sequence starting from exon 1a, and an antisense primer was based on the βgeo coding sequence. Sequence analysis of the PCR product revealed that the insertion occurred between exons 1b and 1c (Fig. 2*A*). As shown in Fig. 1*C*, because at least one part of the first non-coding exon (exon 1a) is used in all transcripts, we hypothesized that the insertion of the βgeo reporter between exons 1b and 1c will interfere with normal splicing and inactivate the *Gabpb2* gene. ES cells were injected into blastocysts to generate GABPβ2$^{+/tp}$ mice. Germline-transmitted GABPβ2$^{+/tp}$ mice gave birth to GABPβ2$^{tp/tp}$ mice at a normal Mendelian ratio and were grossly normal. To determine GABPβ2 protein expression, we raised an antiserum specific for GABPβ2 using its C-terminal portion as an antigen, which can specifically recognize GABPβ2 but does not cross-react with GABPβ1L (Fig. 2, *B* and *C*). By Western blotting, we found that GABPβ2

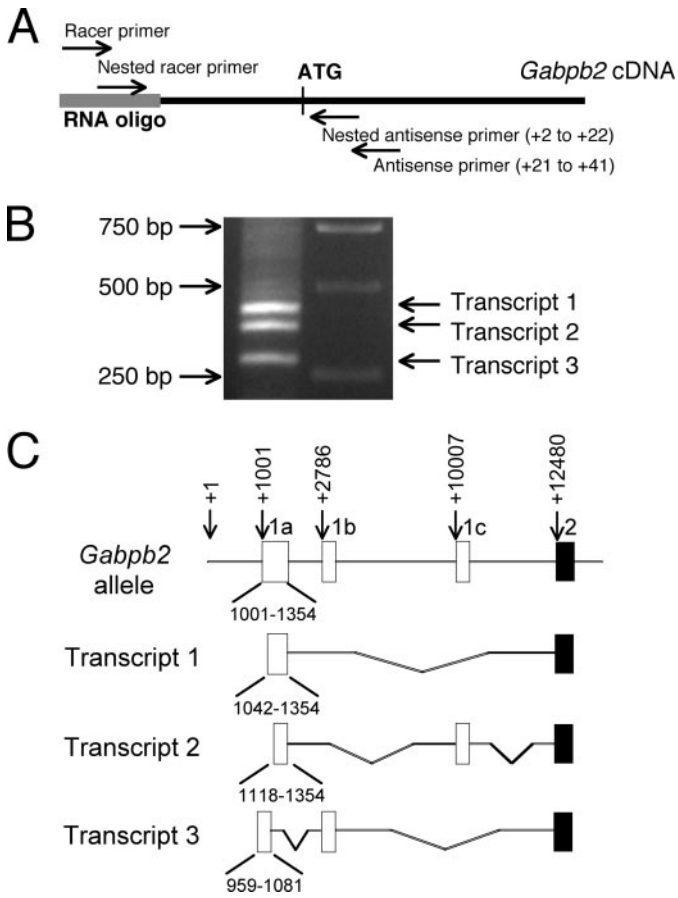

FIGURE 1. **Identification of *Gabpb2* transcription initiation sites and alternative usage of 5′ exons.** *A*, schematic view of the strategy used to identify the 5′-end of GABPβ2 transcripts. Relative locations of the primers are shown. *B*, *Gabpb2* gene is transcribed from three locations. Total RNA was extracted from purified splenic B cells and subjected to 5′-RACE assay. First round PCR was performed using Racer primer and antisense primer, and 1 μl of the PCR product was amplified using nested primers (nested racer primer and nested antisense primer) to increase the amplification specificity. *C*, differential usage of the three 5′ non-coding exons in *Gabpb2* transcripts. The *Gabpb2* gene structure is based on information available at the University of California Santa Cruz (UCSC) genome browser, and the three non-coding exons are defined as 1a, 1b, and 1c. For the purpose of description clarity, a location that is 12,480 bp upstream of the coding exon, *i.e.* exon 2, is arbitrarily defined as "+1," and the location of each non-coding exon is indicated based on the UCSC data base. The actual exon 1a equivalents in each transcript and their relative locations are marked separately.

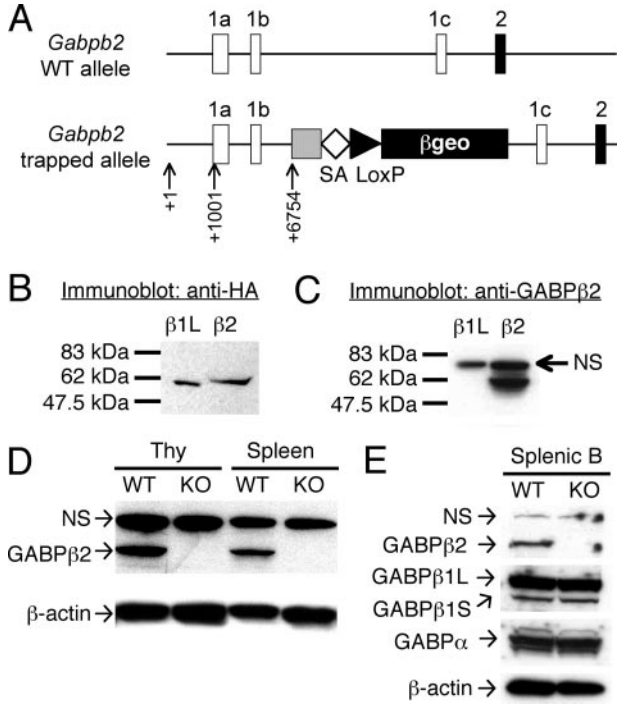

FIGURE 2. **Inactivation of the *Gabpb2* gene using a gene-trapped ES clone.** *A*, mapping of the βgeo insertion in the gene-trapped *Gabpb2* gene. By long template PCR, the insertion of the βgeo reporter gene is mapped between exons 1b and 1c, and the insertion site is at position 6754 based on the location indicated in Fig. 1*C*. The *hatched box* illustrates scrambled sequences at the insertion site, and these sequences are derived from the gene trap vector. *SA*, splice acceptor. *B* and *C*, specificity of anti-GABPβ2 antiserum. 293T cells were transfected with expression plasmids expressing HA-tagged GABPβ1L or GABPβ2, and the cell lysates were fractionated using SDS-PAGE, transferred to membrane, and immunoblotted with an anti-HA antibody (*B*) or anti-GABPβ2 antiserum (*C*). *D*, expression of GABPβ2 in thymocytes and splenocytes. Thymocytes (*Thy*) and total splenocytes were isolated from mice of indicated genotypes. Whole cell lysates were prepared, separated by SDS-PAGE, and blotted with antiserum to GABPβ2 or β-actin (loading control). *KO*, *Gabpb2*$^{tp/tp}$. *NS*, nonspecific bands detected by the antiserum. *E*, expression of GABPβ1L, GABPβ1S, and GABPα in GABPβ2-deficient B cells. Cell lysates from splenic B cells were analyzed for expression of GABP subunits/isoforms using indicated antibodies.

expression is easily detected in both thymocytes and splenocytes in WT mice and is not detectable in GABPβ2$^{tp/tp}$ mice (Fig. 2*D*). This is in contrast to the situation of GABPα$^{tp/tp}$ embryos, which had hypomorphic expression of GABPα protein (14, 16). The difference is likely ascribed to the location of reporter gene insertion and/or the gene context. We further confirmed that ablation of GABPβ2 did not alter the expression of GABPβ1L, GABPβ1S, and GABPα (Fig. 2*E*).

Loss of GABPβ2 Expression Does Not Perturb T and B Cell Development—Previously we have demonstrated that GABPα is critically required for IL-7Rα expression in T cells as well as for normal B cell development (14, 16). T cells develop in the thymus, with the most immature thymocytes being double negative (DN) for CD4 and CD8 expression. DN thymocytes then develop into CD4 and CD8 double positive (DP) cells, which further mature to $CD4^+$ or $CD8^+$ single positive cells (25). The thymocyte, splenocytes, splenic T and B cells numbers between GABPβ2$^{tp/tp}$ and WT control were comparable (Fig. 3*A*). Staining of thymoyctes with anti-CD4 and anti-CD8 antibodies revealed that the percentages of DN, DP, $CD4^+$, and $CD8^+$ subset were not altered in GABPβ2$^{tp/tp}$ mice (Fig. 3*B*). DN thymocytes can be further divided into four developmental stages based on CD25 and CD44 expression, *i.e.* DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$). Fractionation of DN thymocytes with CD25 and CD44 staining showed that all four DN subsets appeared at similar frequencies between WT and GABPβ2$^{tp/tp}$ mice (Fig. 3*C*). These results suggest that the loss of GABPβ2 expression does not affect T cell development, including early stages. In the periphery, splenic $CD4^+$ and $CD8^+$ T cells in GABPβ2$^{tp/tp}$ mice appeared at a normal ratio (Fig. 3*D*), and IL-7Rα expression on GABPβ2$^{tp/tp}$ $CD4^+$ and $CD8^+$ T cells was not diminished (Fig. 3*E* and data not shown for CD8 cells), indicating that GABPβ2 is dispensable for IL-7Rα expression on peripheral T cells.

We also examined B cell development in GABPβ2$^{tp/tp}$ mice. Fractionation of bone marrow cells reveals three populations of sequentially developing B cells, *i.e.* pro-B and pre-B ($B220^+IgM^-$), immature ($B220^{med}IgM^+$), and recirculating B cells ($B220^{high}IgM^+$) (26). All three populations in the bone marrow of GABPβ2$^{tp/tp}$ mice were detected at similar percentages to those observed in WT controls (Fig. 3*F*). In the periphery, the frequency of total splenic B cells and marginal zone B cells ($CD21^{high}CD23^{dim}$) was comparable between WT and GABPβ2$^{tp/tp}$ mice (Fig. 3*H*). The $CD23^{bright}B220^+$ subset is heterogeneous and can be further fractionated to sequentially maturation stages, transitional 1 ($CD24^{high}CD21^{low}$), transitional 2 ($CD24^{high}CD21^{high}$), and mature follicular ($CD24^{dim}CD21^{dim}$) B cells (27), and these maturing B cells showed similar frequency in both WT and GABPβ2$^{tp/tp}$ mice (Fig. 3*I*). These data collectively demonstrate that inactivation of GABPβ2 expression did not detectably perturb B cell development in the bone marrow or further maturation in the spleen.

T and B Cell Proliferation in the Absence of GABPβ2—To determine if mature T and B cells that developed in the absence of GABPβ2 are functional, we isolated splenic T and B cells by negative selection. We stimulated T cells with different doses of plate-bound anti-CD3 in the presence and absence of anti-CD28 mAb and found that both WT and GABPβ2-deficient T cells proliferated similarly (Fig. 4*A*). In contrast, when the purified B cells were stimulated with anti-IgM, which cross-links the B cell receptors (BCRs), GABPβ2-deficient B cells showed increased proliferation in the presence or absence of IL-4 (Fig. 4*B*). However, anti-CD40-elicited proliferation was comparable in both WT and GABPβ2$^{tp/tp}$ B cells (Fig. 4*C*). In addition to the clonally rearranged BCRs, B cells express nonclonal pattern recognition receptors, notably Toll-like receptors (TLRs) including TLR4 and TLR9 (28–30). We used lipopolysaccharide (LPS) to stimulated TLR4, and ODN1826 (a synthetic oligonucleotide containing unmethylated CpG) to stimulated TLR9. WT and GABPβ2-deficient B cells proliferated similarly in response to both stimulants (Fig. 4*D*). These data collectively suggest that B cells lacking GABPβ2 have an enhanced proliferative response specifically to BCR stimulation.

We next tested if the increased B cell proliferation is a result of suppressed apoptosis or increased cell division. We activated B cells with anti-IgM and monitored apoptosis using Annexin V and 7-AAD staining on various days poststimulation. We did not find any consistent differences in percentages of Annexin V^+7-AAD^+

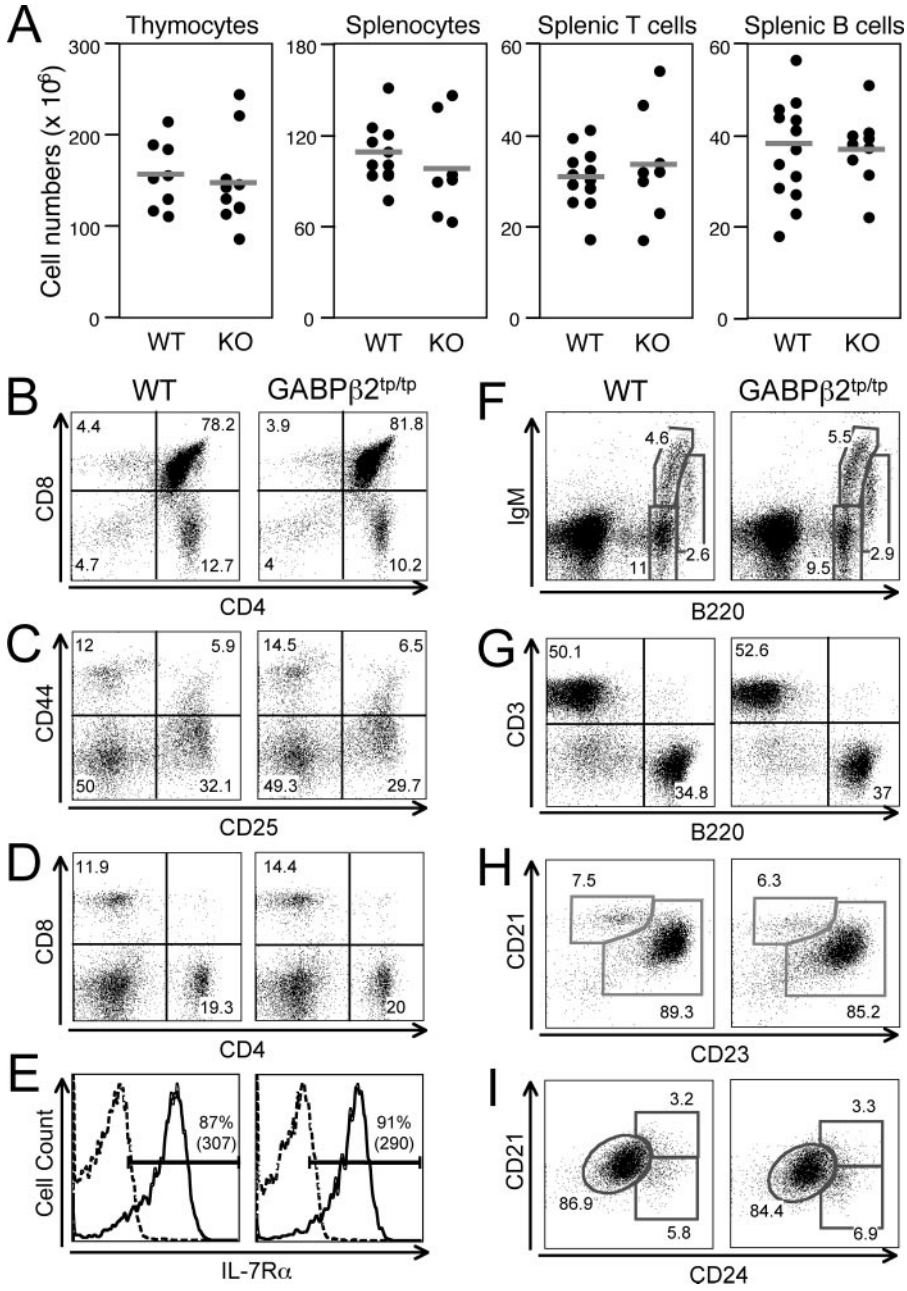

FIGURE 3. **Normal T and B cell development in GABPβ2$^{tp/tp}$ mice.** *A*, cellularity of thymus and spleen. Cell numbers of the thymus and spleen in WT and GABPβ2$^{tp/tp}$ (KO) mice were determined. The splenocytes were stained with anti-CD3 and anti-B220 antibodies, and percentages of CD3$^+$ and B220$^+$ were used to calculated splenic T and B cells, respectively. *B*, thymocyte development. Thymocytes from WT and GABPβ2$^{tp/tp}$ mice were stained with anti-CD4 and anti-CD8 antibodies and the percentage of each population was shown. *C*, early thymocyte developmental stages. The DN thymocytes in *B* were fractionated based on CD25 and CD44 expression. *D*, splenic CD4 and CD8 T cells. *E*, IL-7Rα expression on CD4 T cells. Percentage of IL-7Rα^+ cell and mean fluorescence intensity (*in parentheses*) were shown. *F*, B cell development in the bone marrow. Bone marrow cells were isolated from WT and GABPβ2$^{tp/tp}$ mice and stained with anti-B220 and anti-IgM antibodies. The percentage of each developing B cell population was shown. *G*, splenic T and B cells. *H*, marginal zone and follicular B cells in the spleen. B220$^+$ cells were fractionated based on CD21 and CD23 expression. *I*, maturation stages of follicular B cells. The B220$^+$CD23^{bright} population in *H* was further fractionated based on CD21 and CD24 expression. Representative data from at least five independent experiments are shown.

apoptotic cells (data not shown). In contrast, when labeled with carboxy-fluorescein diacetate succinimidyl ester (CFSE) before anti-IgM stimulation, GABPβ2$^{tp/tp}$ B cells showed moderately accelerated CFSE dilution on all days examined (Fig. 4*E*). These results indicated that BCR-activated B cells can proliferate faster in the absence of GABPβ2.

GABPβ2-deficient B Cells Showed Increased in Vivo Responses to Immunization—To investigate if the increased B cell division is associated with enhanced humoral immune responses, we immunized the mice with both T-independent (TNP-Ficoll) and T-dependent (ovalbumin) antigens. Although TNP-specific IgM and IgG3 were produced at similar levels in WT and GABPβ2-deficient mice (Fig. 5*A*), production of ovalbumin-specific IgM and IgG1 in GABPβ2$^{tp/tp}$ mice was ~1.5-fold of those in littermate controls (Fig. 5*B*). In the immune response to protein antigens, B cells migrate into the follicle in lymphoid organs and form germinal centers (GCs), where antigen-specific B cells further expand and undergo class-switch recombination. To further delineate if loss of GABPβ2 expression can enhance GC responses to protein antigens, we immunized the mice with SRBC and examined GC formation in the spleen. Confocal microscopic analysis showed that both WT and GABPβ2$^{tp/tp}$ mice can form GCs 8 days after immunization, and no apparent alteration in GC size was observed (data not shown). To quantitatively determine the SRBC-elicited GC responses, we used fluorochrome-conjugated PNA, which selectively binds to GC B cells. In most cases, B220$^+$PNA^{high} cells were detected at an increased frequency in GABPβ2$^{tp/tp}$ mice (Fig. 5*C*). On average, we observed a 33 and 40% increase in GC B cell frequency on day 8 and day 12 postimmunization, respectively (Fig. 5*D*). Similarly, the absolute numbers of splenic GC B cells were also increased by 51% on day 8 and by 31% on day 12 (Fig. 5*E*), albeit the difference did not reach statistical significance. These data collectively suggest that GABPβ2-deficient B cells exhibit moderately increased responses to protein antigens.

DISCUSSION

A functional GABPα/β complex requires two subunits, GABPα and GABPβ. The DNA binding Ets domain of GABPα directs the GABP complex to its target genes and determines

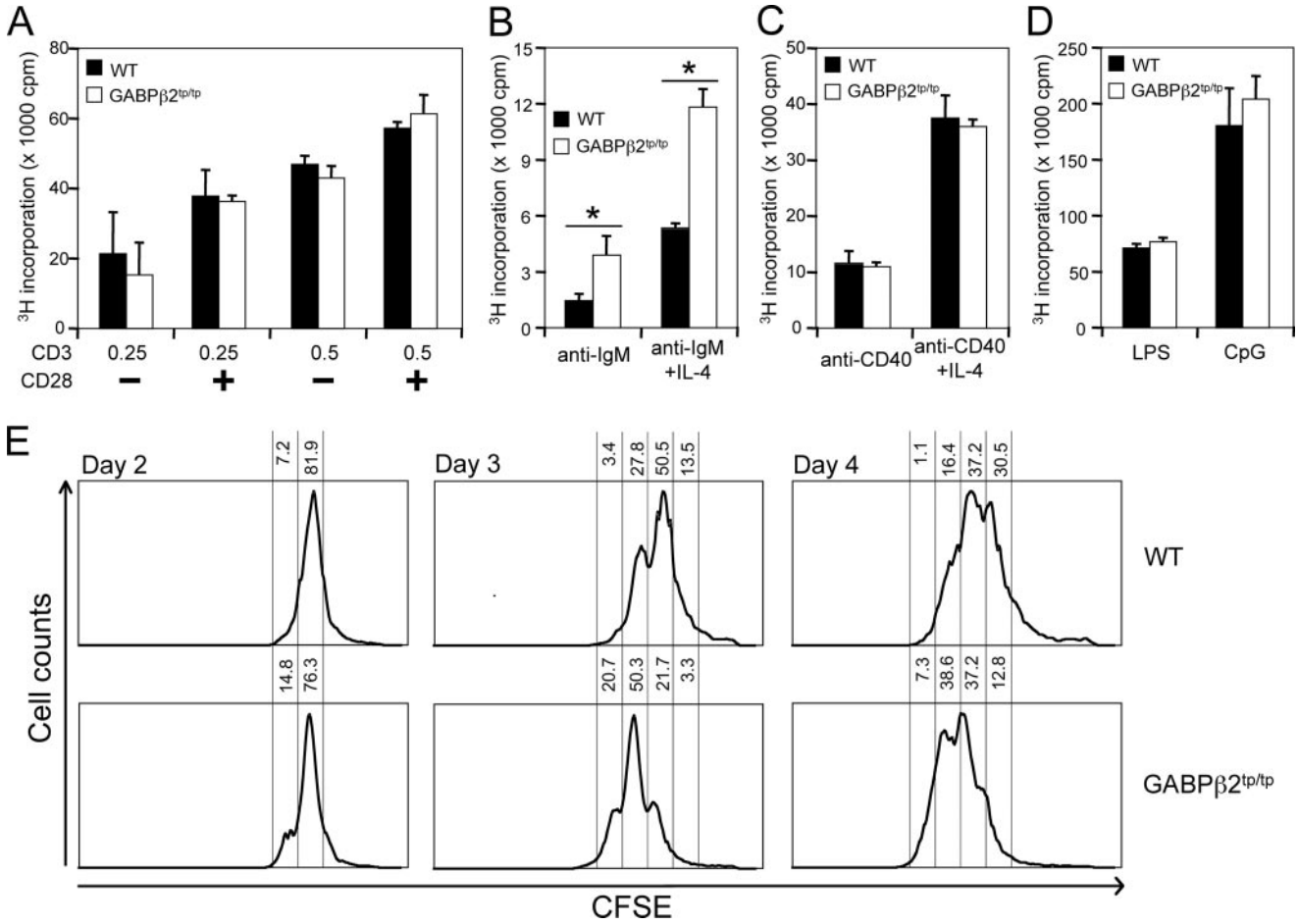

FIGURE 4. **T and B cell proliferation in GABPβ2$^{tp/tp}$ mice.** *A*, T cell proliferation. Splenic T cells were purified by negative selection. The cells (1×10^5 per well) were stimulated with anti-CD3 (0.25 or 0.5 μg/ml) in the presence or absence of anti-CD28 (1 μg/ml) for 60 h, pulsed with 1 μCi of [^{3}H]thymidine, harvested 12 h later, and ^{3}H activity incorporated into cells was determined. *B–D*, B cell proliferation in response to anti-IgM (*B*), anti-CD40 (*C*), and LPS or CpG (*D*). Splenic B cells were purified by negative selection. The cells (0.6×10^5 per well) were activated for 48 h, pulsed and harvested as in *A*. The final concentration of each stimulus is anti-IgM, 2.5 μg/ml, anti-CD40, 5 μg/ml, LPS, 1 μg/ml, CpG (ODN1826), 1 μg/ml, and IL-4, 10 ng/ml. *E*, accelerated cell division of GABPβ2-deficient B cells in response to BCR stimulation. Purified B cells were stained with CFSE, stimulated with anti-IgM (10 μg/ml), and dilution of CFSE was monitored on days 2–4 poststimulation. Percentages at each division are shown on *top* of each panel. Data shown are representative of at least three independent experiments. *Asterisks* in *B* indicate $p < 0.01$ by Student's *t* test.

binding specificity. Activation or repression of GABP target genes is mediated through the C terminus of GABPβ. As revealed in crystal structure studies of GABP, the interaction between GABPα and GABPβ augments and stabilizes the DNA binding (31). In contrast to embryonic lethality caused by ablation of GABPα (4, 14), we show here that mice deficient for GABPβ2 were viable. Previously we have demonstrated that GABPα is required for IL-7Rα expression in peripheral T cells (14) and normal B cell development (16). In GABPβ2-deficient mice, both T and B cells developed normally and the expression of IL-7Rα was not affected. These results indicate that GABPβ2 is dispensable for normal embryogenesis and lymphocyte development. These observations are quite similar to those in GABPβ1L$^{-/-}$ mice as we recently reported (22). Given the structural similarity between GABPβ1L and GABPβ2, loss of one GABPβ isoform is likely compensated for by the other. Indeed, we have shown that both GABPα/GABPβ1L and GABPα/GABPβ2 heterodimers can bind to the Ets motif in the IL-7Rα promoter region (22).

In contrast to the possible redundancy between GABPβ1L and GABPβ2 in lymphocyte development and T cell activation, GABPβ2 appears to have a distinct role in moderately restraining B cell proliferation and humoral responses to protein antigens. GABPβ2-deficient B cells manifested enhanced proliferation specifically by BCR stimulation but not by CD40 ligation or TLR stimulation. This observation suggests that GABPβ2 may have a specific role in negatively regulating BCR signaling, rather than signals that are derived from CD40-CD40 ligand interaction, TLR4 (activated by LPS), or TLR9 (activated by CpG nucleotides). Ligation of BCRs by antigens leads to rapid phosphorylation and activation of Syk, which further activate downstream signal components, including the phosphoinositide 3-kinase (PI3K)/Akt, extracellular signal-regulated kinase (Erk), and nuclear factor-κB (NF-κB) pathways, all three of which are known to be crucial for the survival and proliferation of B cells (32). We probed BCR signaling pathways by stimulating purified B cells with anti-IgM and measuring phosphorylation of tyrosines 519/520 in Syk, serine 473 in Akt, threonine 202 and tyrosine 204 in p44/42 Erk, and serine 32 in IκBα. However, no apparent changes were observed with the potency and duration of these signal pathways, and the protein levels of these signaling molecules were similar between WT and

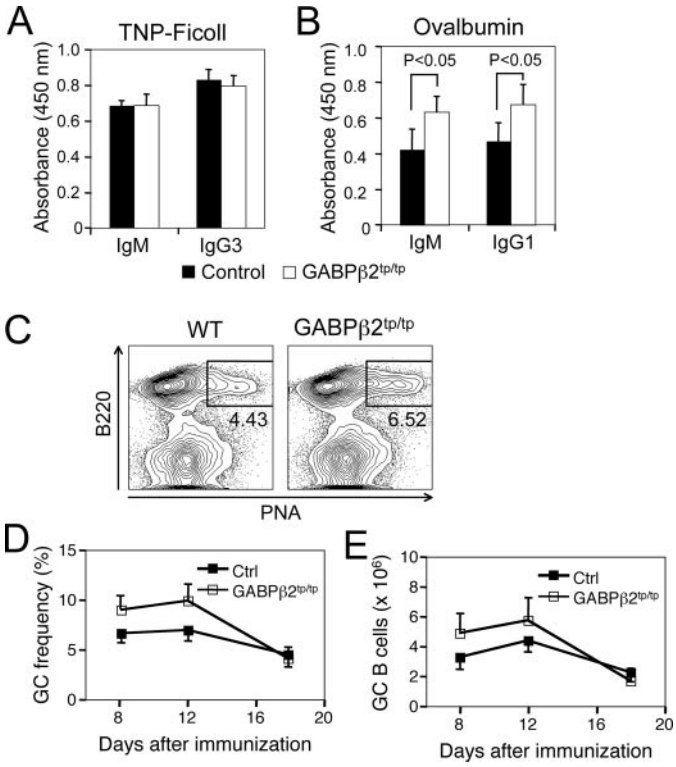

FIGURE 5. **Increased humoral responses in GABP$\beta2^{tp/tp}$ mice.** *A* and *B*, antigen-specific antibody responses. GABP$\beta2^{tp/tp}$ and WT littermate controls were immunized with TNP-Ficoll (*A*) or ovalbumin (*B*), and antigen-specific Ig isotypes were determined by ELISA (16). Data in *B* were statistically analyzed using the Student's *t* test ($n = 5$). *C–E*, germinal center responses. Mice were immunized with SRBC, and spleens were harvested on 8, 12, and 18 days later. *C*, flow cytometric analysis of GC B cells. Spleens were harvested 8 days after SRBC immunization, and the splenocytes were stained with B220 and FITC-conjugated PNA. Data are representative of two independent experiments, with triplicate or quadruplicate samples analyzed. *D* and *E*, kinetic analysis of SRBC-elicited GC responses. Spleens were harvested from SRBC-immunized mice on indicated days and stained for GC B cells as in *C*. Frequency of GC B cells in total splenic B cells and total GC B cell numbers are shown in *D* and *E*, respectively. Data are pooled results from two independent experiments on 6–7 mice. All values are means ± S.E.

GABPβ2-deficient B cells (data not shown). The precise molecular mechanism by which GABPβ2 negatively regulates BCR signaling awaits further investigation.

Consistent with increased BCR-stimulated proliferation of GABPβ2-deficient B cells *in vitro*, GABPβ2-deficient mice displayed increased IgM and IgG1 antibody levels after ovalbumin immunization and a heightened GC response after challenge with SRBC. The increase is somewhat moderate, and a possible explanation is that only BCR-elicited responses were enhanced in the absence of GABPβ2, which may be blunted by similar responses derived from CD40-CD40L interaction. It is noteworthy that mice lacking GABPβ2 did not show apparent difference in antibody levels after a challenge with TNP-Ficoll. This can be explained by the somewhat modest proliferation of B cells observed *in vivo* after immunization with a T-independent polysaccharide antigen, and the restriction of expansion to the first few days after exposure (33). In contrast, antigen-selected B cells undergo marked proliferation for extended periods in GCs after T-dependent challenge (34), allowing for a longer period of time during which the absence of GABPβ2 can generate a higher number of antigen-specific clones. In summary, our data revealed that GABPβ2 has both redundant and distinct roles in the immune system. Lack of GABPβ2 did not affect B cell development but showed increased B cell proliferation and humoral responses to protein antigens. Thus, manipulation of GABPβ2 expression may be a useful approach to modulate B cell responses without interfering with normal B cell development.

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