Critical Role of B Cell Lymphoma 10 in BAFF-Regulated NF-κB Activation and Survival of Anergic B Cells

Mei Yu, Yuhong Chen, Yinghong He, Andrew Podd, Guoping Fu, Jacqueline A. Wright, Eden Kleiman, Wasif N. Khan, Renren Wen and Demin Wang

*J Immunol* published online 19 October 2012
http://www.jimmunol.org/content/early/2012/10/19/jimmunol.1102952
Critical Role of B Cell Lymphoma 10 in BAFF-Regulated NF-κB Activation and Survival of Anergic B Cells

Mei Yu,*† Yuhong Chen, † Yinghong He,*⋆† Andrew Podd, †‡ Guoping Fu, † Jacqueline A. Wright, ‡ Eden Kleiman, ‡ Wasif N. Khan, ‡ Renren Wen, † and Demin Wang*†,‡

Anergy is a key physiological mechanism for restraining self-reactive B cells. A marked portion of peripheral B cells are anergic B cells that largely depend on BAFF for survival. BAFF activates the canonical and noncanonical NF-κB pathways, both of which are required for B cell survival. In this study we report that deficiency of the adaptor protein B cell lymphoma 10 (Bcl10) impaired the ability of BAFF to support B cell survival in vitro, and it specifically increased apoptosis in anergic B cells in vivo, dramatically reducing anergic B cells in mice. Bcl10-dependent survival of self-reactive anergic B cells was confirmed in the Ig hen egg lysozyme/soluble hen egg lysozyme double-transgenic mouse model of B cell anergy. Furthermore, we found that BAFF stimulation induced Bcl10 association with IκB kinase β, a key component of the canonical NF-κB pathway. Consistently, Bcl10-deficient B cells were impaired in BAFF-induced IκBo phosphorylation and formation of nuclear p50/c-Rel complexes. Bcl10-deficient B cells also displayed reduced expression of NF-κBp105/p100, severely reducing BAFF-induced nuclear accumulation of noncanonical p52/RelB complexes. Consequently, Bcl10-deficient B cells failed to express Bcl-xL, a BAFF-induced NF-κB target gene. Taken together, these data demonstrate that Bcl10 controls BAFF-induced canonical NF-κB activation directly and noncanonical NF-κB activation indirectly. The BAFF-R/Bcl10/NF-κB signaling axis plays a critical role in peripheral B cell tolerance by regulating the survival of self-reactive anergic B cells.

The Journal of Immunology, 2012, 189: 000–000.

© 2012 by The American Association of Immunologists, Inc. 0022-1767/12/$16.00

Newly produced B cells, 50–75% of which are estimated to be self-reactive B cells (1, 2), undergo negative selection by three distinct mechanisms in the bone marrow: receptor editing, clonal deletion, and anergy (3–8). The signaling threshold of a self-reactive BCR has long been thought to determine the negative selection mechanism. A highly self-reactive BCR initiates new rearrangements of V genes to change receptor specificity by receptor editing (9–13), or it drives B cells to undergo apoptosis and be eliminated by clonal deletion (14, 15). A moderately self-reactive BCR drives B cells to become unresponsive to Ags, a state termed anergy (16, 17). After emerging from the bone marrow, transitional B cells undergo further maturation and selection in the periphery. Highly self-reactive B cells are removed by clonal deletion, whereas moderately self-reactive B cells become anergic (11, 18). As much as 5–10% of peripheral B cells are anergic, and given the fast turnover rate of these cells, anergy likely represents a major physiological mechanism for establishing immune tolerance (19). In the periphery, although BCR signaling is required for the maintenance of normal and self-reactive anergic B cells (20, 21), TNF family member BAFF is essential for the survival of these B cells (22–24). Importantly, self-reactive anergic B cells have increased dependence on BAFF for survival compared with normal B cells (24, 25). Excess BAFF mainly rescues self-reactive anergic B cells from competitive elimination (24, 25). Thus, in addition to BCR, BAFF plays a crucial role in sustaining the survival of anergic B cells in the periphery.

BAFF supports the survival of B cells through its cognate receptor. Although BAFF binds to three receptors, that is, BAFF-R, B cell maturation Ag, and TACI, BAFF-R plays the predominant role in BAFF function (22, 23). Deficiency of BAFF-R impairs the survival of peripheral B cells similar to deficiency of BAFF, resulting in nearly complete loss of peripheral B cells (22, 26, 27). In contrast, deficiency of B cell maturation Ag impairs the survival of long-lived plasma cells (28), whereas lack of TACI leads to a 2- to 3-fold increase in mature B cell number (29).

One important signaling event of BAFF-R that leads to B cell survival is the NF-κB–dependent upregulation of Bcl-2 family prosurvival members (22, 30). NF-κB/Rel family of transcription factors consists of five members, that is, RelA/p65, c-Rel, RelB, NF-κB1 (p105/p50), and NF-κB2 (p100/p52), which can form homo- and heterodimers (31, 32). NF-κB dimers exist as inactive complexes bound to the inhibitor of NF-κB (IκB), such as IκBα or IκBβ, in resting cells (33). There are two major signaling pathways that mediate the activation of NF-κB, known as the canonical and noncanonical pathways (34). It is known that BAFF-R is able to induce NF-κB activation through both pathways (35–40).

Upon BAFF binding, BAFF-R induces a cellular inhibitor of apoptosis 1/2 and TNFR-associated factor 2 ubiquitin ligase.
complex-dependent degradation of TNFR-associated factor 3, preventing NF-κB–inducing kinase degradation and resulting in the subsequent activation of the noncanonical NF-κB pathway (37, 38). NF-κB–inducing kinase activates IkB kinase (IKKα), and activated IKKα phosphorylates p100, leading to processing of NF-κB2 from a p100 precursor to a p52 product. Then p52 dimerizes with RelB to form the p52/RelB active heterodimer that translocates to the nucleus and regulates gene expression (35, 36). Additionally, BAFF-R can activate NF-κB through the canonical pathway that involves the activation of the IKK complex consisting of IKKα, IKKβ, and NEMO (39, 40). Activated IKK complex induces the phosphorylation and subsequent degradation of IkBα. Removal of IkBs results in the nuclear translocation of p50-containing NF-κB heterodimers and subsequent initiation of gene transcription. Despite progress in this area of research, the mechanisms by which BAFF-R mediates the activation of NF-κB in both the canonical and noncanonical pathways are not fully understood.

B cell lymphoma 10 (Bcl10) is an adaptor protein characterized by an N-terminal caspase recruitment domain and a C-terminal Ser/Thr-phosphorylated domain. Bcl10 plays a critical role in TCR-, FcγR-, and TLR4-induced NF-κB activation. The carboxy-terminal of NF-κB complex-mediated transcription involves the activation of the IKK complex (48–51). Bcl10 deficiency impairs BCR-induced NF-κB activation (47). Additionally, Bcl10 plays a critical role in TCR-, FceR, and TLR4-induced NF-κB activation, and its deficiency impairs NF-κB activation by these receptors (46). Although Bcl10 plays a critical role in NF-κB activation by multiple receptors, its role in BAFFR-mediated NF-κB activation is not known.

Our current studies find that Bcl10 directly controls BAFF-mediated canonical NF-κB activation and induces the expression of NF-κB2/p100, thus indirectly regulating BAFF-mediated noncanonical NF-κB activation. This BAFFR/Bcl10/NF-κB signaling axis specifically supports the survival of self-reactive anergic B cells in vivo. Impairment of this axis by Bcl10 deficiency alters the peripheral tolerance mechanism for self-reactive B cells, switching from anergy to deletion.

Materials and Methods

Mice

Bcl10-deficient mice have been previously described (47). Heterozygous Bcl10-deficient mice were bred to generate wild-type control and Bcl10-deficient mice. Ig hen egg lysozyme (IgHEL) transgenic mice (C57BL/6 MD4) and soluble HEL (sHEL) transgenic mice (C57BL/6 ML5) were obtained from The Jackson Laboratory. These transgenic mice were bred with heterozygous Bcl10+/- mice to generate wild-type IgHEL, Bcl10-deficient IgHEL, wild-type IgHEL, sHEL, and Bcl10-deficient IgHEL, sHEL mice. Mice used for the experiment were generally 2–4 mo old except where specifically indicated. All mouse procedures were approved by the Institutional Animal Care and Use Committee.

Flow cytometry

Single-cell suspensions from the spleen of the experimental and control mice were treated with Gey’s solution to lyse RBCs and resuspended in PBS with 2% FBS. The cells were then stained with a combination of fluorescence-conjugated Abs. PE-Cy7–conjugated anti-CD19 (2D19-0193), allophycocyanin-conjugated anti-IgM (17-5790), FITC-conjugated anti–BAFF-R (11-5943), Cy-Chrome–conjugated anti-B220 (15-0452), PE–conjugated anti-B220 (12-0452), biotin-conjugated anti–AA4.1 (CD93; 13-5892), PE–conjugated anti–CD23 (12-0232), and PE–Cy7–conjugated streptavidin (25-4317) were purchased from eBioscience. PE–conjugated anti-CD11c (11-0109) and FITC-conjugated anti-CD4 (14-0042) were purchased from SouthernBiotech. Allophycocyanin-conjugated streptavidin (554067) was purchased from BD Pharmingen. Stained cells were analyzed on a BD LSR II cytometer with BD FACS Diva software.

B cell isolation

The splenic CD93–immature (also known as AA4.1+) and CD93+ (AA4.1+) mature B cells were isolated from wild-type and Bcl10-deficient mice as previously described (52). Briefly, splenic B cells were isolated by negative selection using anti-CD4–, anti–CD8–, and anti–CD11b-coated MACS microbeads (Miltenyi Biotech) and then stained with biotin-conjugated anti–CD3 (eBioscience). Immature B cells (CD93–) were isolated using streptavidin-conjugated microbeads with the flow through constituting the mature B cells (CD93+). For NF-κB2/p100 mRNA expression studies, mature B cells were also isolated from CD93-depleted splenocytes by positive selection using biotin-conjugated anti–CD23 (eBioscience) Abs and streptavidin-conjugated microbeads. Purity of CD93+ and CD93– CD23+ B cells was ~90 and 95%, respectively, as determined by anti-B220 Ab staining followed by flow cytometry.

Immunoprecipitation and Western blotting analysis

Splenic AA4.1+ mature B cells (1 × 106) from wild-type and Bcl10-deficient mice were stimulated with BAFF (100 ng/ml) at 37°C for the indicated times and then lysed in lysis buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 3 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin). Cell lysates were subjected to Western blotting analysis with the indicated Abs. For detecting IkB phosphorylation, splenic mature B cells were pretreated with MG132 for 30 min before BAFF stimulation. For immunoprecipitation, wild-type AA4.1+ mature B cells (2 × 106) were stimulated with BAFF (200 ng/ml) or anti-IgM (10 μg/ml) for the indicated times. Cell lysates were precleared with Sepharose beads and subsequently incubated with anti-Bcl10 Ab-conjugated Sepharose beads (sc-5273 AC; Santa Cruz Biotechnology) at 4°C overnight. After washing five times with cold lysis buffer, bound proteins were eluted in 2× Laemmli buffer at 95°C for 5 min and subjected to 10% SDS-PAGE, followed by Western blotting analysis with the indicated Abs.

Rabbit polyclonal anti-ERK (sc-903), anti-p50 (sc-114), anti-p65 (sc-109), anti–c-Rel (sc-371), anti-IκBα (sc-226), and mouse monoclonal anti-p52 (sc-7386), anti–phospho-ERK (pThr202/pTyr204, sc-7383), anti-Bcl10 (sc-5273), and anti–YY1 (sc-7341) were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti–phospho-IκBα (Ser276/286, 9246), rabbit polyclonal anti–phospho-Akt (phospho-Ser73, 9271) and anti-IκBα (9242), and rabbit monoclonal anti–IKKβ (2C8, 2370) were purchased from Cell Signaling Technology. Rabbit polyclonal anti–Bcl10 (B22630) was purchased from BD Transduction Laboratories. Hamster monoclonal anti–MAL1 was a generous gift from Dr. Vishva Dixit (Genentech, San Francisco, CA). Mouse monoclonal anti–phospho-IκBα (Ser276/286, 9246), rabbit polyclonal anti–phospho-Akt (phospho-Ser73, 9271) and anti–IkBα (9242), and rabbit monoclonal anti–IKKβ (2C8, 2370) were purchased from Cell Signaling Technology. Rabbit polyclonal anti–Bcl10 (B22630) was purchased from BD Transduction Laboratories. Hamster monoclonal anti–GADPH (MAB374), anti–Bcl10 (AB16506), and anti–actin (MAB1501R) were purchased from Chemicon International/Millipore.

NF-κB gel mobility shift assay

Splenic CD93– mature B cells (1 × 106) from wild-type and Bcl10-deficient mice were stimulated with BAFF (200 ng/ml) or 3 or 16 h and then lysed in the lysis buffer (20 mM HEPES pH 7.9, 350 mM NaCl, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 20% glycerol, 1% NP-40). Cell lysates were incubated with 32P-labeled NF-κB probe (5'-AGTTGAGGGACATTCCC-CAGGC-3', Santa Cruz Biotechnology) for 15 min at room temperature, resolved on a 4% polyacrylamide gel at 4°C, and exposed to x-ray film. For supershift, cell lysates were incubated with the indicated Abs for 15 min before adding the probes.

Preparation of cytoplasmic and nuclear extracts

Splenic CD93– mature B cells (2 × 106) from wild-type and Bcl10-deficient mice were stimulated with BAFF (100 ng/ml) for 16 h. The cells were collected and suspended in 100 μl cold buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.2 mM EDTA, 1 mM DTT, 3 μg/ml aprotinin, 2 μg/ml pepstatin, and 1 μg/ml leupeptin). After incubation on ice for 15 min, Nonidet P-40 was added to a final concentration of 0.5%. The mixtures were vortexed for 10 s and spun at 16,000 × g for 30 s. The supernatants were collected as the cytoplasmic extracts. The pellets were washed with buffer A once and resuspended in 50 μl buffer B (20 mM HEPES [pH 7.9], 400 mM NaCl, 2 mM EDTA, 1 mM DTT, 3 μg/ml aprotinin, 2 μg/ml pepstatin, and 1 μg/ml leupeptin), followed by incubation on ice for 15 min. The mixtures were spun at 16,000 × g for 5 min and the supernatants were collected as the nuclear extracts.

Quantitative RT-PCR

Splenic CD93–CD23+ mature or CD93– immature B cells from wild-type and Bcl10-deficient mice were stimulated with BAFF (100 ng/ml) for 4 or 16 h. RNA was extracted from the cells using an RNeasy Mini kit (Qiagen).
and quantified. Equal amounts of mRNA were used to generate cDNA using a QuantiTect reverse transcription kit (Qiagen). Real-time PCR was performed with NF-κB2/p100 primers (Mm00479807), other FAM-labeled probes, and TaqMan Universal Master mix using a StepOne real-time PCR system (Applied Biosystems). The relative p100 mRNA fold induction was calculated relative to 18S rRNA.

Propidium iodide staining assay
Splenic AA4.1+ mature or AA4.1+ immature B cells from wild-type or Bcl10-deficient mice were cultured at a density of 2 × 10^5 cells/ml with or without BAFF (100 ng/ml) for the indicated times, followed by propidium iodide staining and FACS analysis.

BrdU incorporation assay
The in vivo BrdU labeling assay was performed as described (47). In brief, mice were injected i.p. with 1 mg BrdU (Sigma-Aldrich) in 0.2 ml PBS at 12-h intervals for 4 d. Splenocytes from BrdU-treated mice were stained with anti-B220. Cells were then fixed and stained with anti-BrdU according to the manufacturer’s instructions of an FITC BrdU flow kit (559619; BD Pharmingen). The degree of BrdU positivity in the gated B cells was analyzed by FACS.

TUNEL assay
Splenic B220+CD93+CD23-IgMlo) populations (19). Previous studies have shown that the survival function of BAFF is particularly important for self-reactive anergic B cells (22–25). Based on our finding that Bcl10-deficient B cells survived poorly in re-

Results

Bcl10 deficiency severely impairs BAFF-mediated B cell survival
All peripheral B cells require signals from both the BCR and BAFF-R for their survival (20–25). Self-reactive anergic B cells display even greater dependence on BAFF-R signaling for their survival (25). In present study, we examined the role of Bcl10 in BAFF-mediated B cell survival. Splenic CD93+ B cells, which contain T1, T2, and An1 anergic subpopulations, and CD93− B cells, which are mature follicular (FO) and marginal zone B cells, were isolated from wild-type and Bcl10-deficient mice. The cells were cultured in the absence or presence of BAFF, and cell viability was determined at various time points. In the absence of BAFF, both wild-type and Bcl10-deficient CD93+ and CD93− B cells underwent apoptosis over time, although more mutant cells than corresponding wild-type cells died after the initial time point (Fig. 1). As expected, addition of BAFF markedly rescued both wild-type CD93+ and CD93− B cells from apoptosis (Fig. 1). Importantly, CD93+ B cells from Bcl10-deficient mice displayed no survival response to BAFF (Fig. 1, right). Although BAFF increased viability of Bcl10-deficient CD93+ B cells, this was markedly reduced compared with wild-type CD93+ B cells (Fig. 1, left). Thus, BAFF-mediated immature B cell survival is entirely Bcl10-dependent, whereas mature B cells only partially depend on Bcl10 for their survival.

Bcl10 deficiency causes drastic reduction in anergic B cells
Prior studies have shown that the survival function of BAFF is particularly important for self-reactive anergic B cells (22–25). Based on our finding that Bcl10-deficient B cells survived poorly in response to BAFF, we examined the effects of loss of Bcl10 on the survival of anergic B cell population. In the spleen, B220−CD93− cells are largely mature FO B cells (B220−CD93+CD23+IgMlo), whereas B220−CD93− cells contain T1 (B220−CD93+CD23−IgMlo), T2 (B220−CD93−CD23+IgMlo), and T3-type An1 anergic (B220− CD93−CD23+IgMlo) populations (19). Previous studies have shown that FO B cells were reduced in the spleens derived from Bcl10-deficient relative to wild-type mice (47). In this study we found that T1 B cells were slightly increased and T2 B cells were markedly increased in the spleens derived from Bcl10-deficient mice relative to those from wild-type animals (Fig. 2A, 2B). In contrast, the percentage and number of splenic An1 anergic B cells (B220− CD93−CD23−IgMlo) was drastically reduced in Bcl10-deficient relative to wild-type mice (Fig. 2A–C). Thus, Bcl10 deficiency results in a drastic reduction in self-reactive anergic B cells.

FIGURE 1. Bcl10 deficiency impairs the survival of peripheral B cells in response to BAFF. CD93+ (AA4.1+) and CD93− (AA4.1−) B cells from wild-type (+/+ ) and Bcl10-deficient (−/−) mice were cultured in the absence or presence of BAFF. At the indicated time points, cell survival rates were determined by propidium iodide staining. Data are representative of five independent experiments.

Bcl10 deficiency specifically increases anergic B cell apoptosis in vivo
Next, we examined the rate of apoptosis in mutant An1 anergic B cells. We stained splenocytes from wild-type or Bcl10-deficient mice with anti-B220, anti-CD93, anti-IgM, and anti-CD23 and then analyzed them for apoptosis by TUNEL assay. Bcl10-deficient An1 anergic B cells had an increased rate of apoptosis relative to that of the corresponding wild-type B cells (Fig. 2D). In contrast, Bcl10-deficient T1, T2, and FO B cells had similarly low rates of apoptosis relative to those of the corresponding wild-type B cell subpopulations, as previously reported (Fig. 2D) (47). These data demonstrate that Bcl10 deficiency specifically impairs the survival of An1 anergic B cells in vivo.

Reduced Bcl10-deficient anergic B cells in IgHEL−sHEL mouse model
To further investigate a role of Bcl10 in BAFF-mediated survival of An1 anergic B cells, Bcl10-deficient mice were crossed with IgHEL− transgenic mice, which bear rearranged H and Igκ L chain genes encoding a BCR that specifically recognizes HEL (16). sHEL induces wild-type IgHEL transgenic B cells to become anergic (16, 19). In the absence of self-Ag sHEL, the spleens from Bcl10-deficient IgHEL transgenic mice displayed a slight reduction of total splenic B cells compared with wild-type controls (Fig. 3A, 3B). FACS analysis of splenocytes with B220, CD93, IgM, and CD23 staining showed that the spleens from both wild-type and Bcl10-deficient IgHEL transgenic mice had a large population of FO mature B cells, a small population of T1 B cells, a moderate population of T2 B cells, and no An1 anergic B cells (Fig. 3C). As expected, chronic exposure of wild-type IgHEL B cells to sHEL induced self-reactive transgenic B cells into An1 anergic B cells (Fig. 3C, second row) and reduced the population of splenic B cells in wild-type IgHEL−sHEL relative to wild-type IgHEL transgenic mice (Fig. 3A, 3B). In contrast, in the absence of Bcl10, sHEL drastically reduced splenic B cells, especially An1 anergic B cells, in the spleens of Bcl10-deficient relative to wild-type IgHEL−sHEL mice (Fig. 3). These results confirm that Bcl10 deficiency results in a drastic reduction of An1 anergic B cells in the well-defined IgHEL−sHEL transgenic model of B cell anergy.

The severe reduction of the An1 anergic B cells in Bcl10-deficient IgHEL−sHEL mice led us to examine whether the reduction is due to
increased apoptosis of the mutant cells. TUNEL assay demonstrated that the apoptosis rates of splenic B cells were markedly increased in Bcl10-deficient relative to wild-type Ig^HEL^xHEL^ double-transgenic mice (Fig. 4A, 4B). Of note, the apoptosis rates of splenic B cells from both wild-type and mutant Ig^HEL^ single-transgenic mice were equally low (Fig. 4A, 4B).

We also examined whether impaired cell proliferation contributes to the drastic reduction of anergic B cells in mutant Ig^HEL^xHEL^ mice. In vivo BrdU labeling assay demonstrated that the BrdU labeling rates of splenic B cells were reduced in Bcl10-deficient relative to wild-type Ig^HEL^ single-transgenic mice (Fig. 4C, 4D). However, the in vivo BrdU labeling rates of splenic B cells were comparable between Bcl10-deficient and wild-type Ig^HEL^xHEL^ double-transgenic mice (Fig. 4C, 4D). Of note, splenic B cells in Bcl10-sufficient and Bcl10-deficient Ig^HEL^xHEL^ double-transgenic mice were mainly anergic (Fig. 3C). Compared to wild-type double-transgenic mice, mutant double-transgenic mice had a dramatic reduction of these anergic cells (Fig. 3A, 3C). Thus, Bcl10 deficiency mainly impairs the in vivo survival of anergic B cells in Ig^HEL^xHEL^ transgenic mice. Overall, these data demonstrate that in the absence of self-Ag, Bcl10-deficient peripheral B cells can usually survive in vivo, whereas in the presence of self-Ag, self-reactive Bcl10-deficient B cells undergo apoptosis instead of anergy.

**Bcl10 deficiency impairs BAFF-induced canonical NF-κB activation**

Owing to a critical role for BAFF in anergic B cell survival, Bcl10-dependent mechanisms that contribute to BAFF-R signaling were investigated. Bcl10 deficiency could reduce the expression of BAFF-R on B cells, resulting in an impairment of the ability of BAFF to support B cell survival. To examine this possibility, we compared Bcl10-deficient mice. Data shown are obtained from at least seven (A, B, D) or four (C) mice in each group. Error bars show ± SD. *p < 0.01, **p = 0.02.

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Marked reduction of anergic B cells in Bcl10-deficient mice and increased apoptosis of the mutant anergic B cells. (A) Splenic B cell subpopulations. Splenocytes from wild-type and Bcl10-deficient mice were stained with Abs to B220, CD93, IgM, and CD23. In B220^CD93^ gated cells, T1 (CD23^IgM^), T2 (CD23^IgM^), and An1 anergic (CD23^IgM^) B cells are shown. Percentages indicate B cells in the gated B220^+^ population. (B) Bar graphs show the percentages of T1, T2, and An1 anergic B cells in the gated B220^+^ population. (C) Bar graphs show the numbers of An1 anergic B cells in the spleens of wild-type and Bcl10-deficient mice. (D) Apoptosis of splenic B cell subpopulations. Bar graphs show the degree of TUNEL labeling in T1, T2, An1 anergic, and FO B cells of wild-type and Bcl10-deficient mice. Data shown are obtained from at least seven (A, B, D) or four (C) mice in each group. Error bars show ± SD. *p < 0.01, **p = 0.02.

Furthermore, we investigated whether Bcl10 is required for BAFF-induced activation of the canonical NF-κB by gel mobility shift assays. As previously reported (39, 40), canonical NF-κB activation by BAFF stimulation occurred at an early time point (Fig. 5D) and involved the formation of p50/p100, which is a critical substrate in BAFF-mediated NF-κB activation (Fig. 5E). Taken together, these data demonstrate that Bcl10 is critical for BAFF-induced canonical NF-κB activation in B cells.

**Bcl10 deficiency indirectly impairs BAFF-induced noncanonical NF-κB activation**

Previous studies have shown that BCR-mediated canonical NF-κB activity contributes to the basal as well as induced expression of NF-κB2/p100, which is a critical substrate in BAFF-mediated noncanonical NF-κB activation and B cell survival (55, 56).
Bcl10 deficiency disrupts BCR-mediated NF-κB activation (46, 47). Our Western blot analysis of the expression of the NF-κB family members revealed that the basal level of NF-κB2/p100, but not other NF-κB members, was reduced in Bcl10-deficient relative to wild-type splenic mature B cells (Fig. 6A). However, the NF-κB2/p100 protein was efficiently processed into p52 in the presence of BAFF in both wild-type and Bcl10-deficient mature B cells, as indicated by reduction in NF-κB2/p100 protein (Fig. 6B). Consistent with reduced NF-κB2/p100 protein in the Bcl10-deficient B cells, BAFF-induced NF-κB2/p100 mRNA expression was also reduced in Bcl10-deficient relative to wild-type immature and mature B cells (Fig. 6C). Thus, lack of Bcl10 reduces basal and BAFF-induced expression of NF-κB2/p100 in B cells. This limits the p100 substrate necessary for the optimal activation of noncanonical NF-κB in Bcl10-deficient B cells.

It is well established that constant BAFF exposure induces noncanonical NF-κB activation through a relatively well-defined pathway that requires processing of NF-κB2/p100 into p52. The p52 then dimerizes with RelB to form p52/RelB heterodimer (35, 36). Our results showed that induction of NF-κB DNA binding activity by constant BAFF exposure was severely reduced in Bcl10-deficient relative to wild-type B cells (Fig. 6D). These NF-κB DNA–binding complexes in wild-type B cells were supershifted strongly by anti-p50 and slightly by anti–c-Rel or anti-RelB, whereas the supershift with anti-RelA/p65 or anti-p52 was least prominent (Fig. 6E). However, anti-RelA/p65 and anti-p52 reduced intensity of BAFF-induced NF-κB bands (Fig. 6E). These results suggest that in addition to the expected p52/RelB heterodimer (35, 36), p50/RelA and p50/c-Rel heterodimers were also formed in response to BAFF.

FIGURE 3. Dramatic reduction of Bcl10-deficient anergic B cells in Ig^HEL^IgHEL mouse model. Splenocytes from Bcl10^+/+^IgHEL, Bcl10^−/−^IgHEL, Bcl10^+/+^IgHEL^shHEL, and Bcl10^−/−^IgHEL^shHEL transgenic mice were stained with Abs to B220, IgM, CD93, and CD23. (A) FACS analysis with B220 and IgM staining of lymphocytes. Percentages indicate B220^+^ cells in the gated lymphoid populations. (B) Bar graphs show the percentages (upper) and numbers (lower) of total splenic B cells in the spleens of the indicated mice. (C) Splenic B cell subpopulations. Upper, FACS analysis with B220 and CD93 staining of lymphocytes; middle, FACS analysis with IgM and CD23 staining of B220^+^CD93^−^-gated cells; lower, FACS analysis with IgM and CD23 staining of B220^+^CD93^−^-gated cells. Percentages indicate cells in the gated lymphoid populations. (D) Bar graphs show the numbers of CD93^−^An1 anergic B cells in the spleens of the indicated mice. Data shown are obtained from 11 (A, B), 3 (C), or 4 (D) mice in each group. Error bars show ± SD.

* p < 0.01, ** p = 0.01.

FIGURE 4. Increased apoptosis and normal proliferation of anergic B cells in Bcl10-deficient IgHEL^shHEL mice. (A) TUNEL labeling of splenic B cells. Splenocytes from Bcl10^+/+^IgHEL, Bcl10^−/−^IgHEL, Bcl10^+/+^IgHEL^shHEL, and Bcl10^−/−^IgHEL^shHEL transgenic mice were stained with anti-B220 Abs. Then, the degree of TUNEL labeling in B220^+^ cells was determined by FACS analysis. Percentages indicate TUNEL^+^ cells in the gated B220^+^ cells. (B) Statistical analysis of the percentages of TUNEL^+^ cells from (A). (C) BrdU incorporation in splenic B cells. BrdU was injected into Bcl10^+/+^IgHEL, Bcl10^−/−^IgHEL, Bcl10^+/+^IgHEL^shHEL, and Bcl10^−/−^IgHEL^shHEL transgenic mice. Splenocytes from the mice were stained with anti-B220 Abs, followed by BrdU staining. Percentages indicate BrdU^+^ cells in the gated B220^+^ cells. (D) Statistical analysis of the percentages of BrdU^+^ cells from (C). Data shown are obtained from at least five (A, B) or seven (C, D) mice in each group. Error bars show ± SD. *p < 0.01.
In contrast, the NF-κB DNA-binding complexes in Bcl10-deficient B cells were supershifted or reduced in intensities by all Abs used except anti-p52 (Fig. 6E). These results are consistent with reduced canonical NF-κB activation (p50/RelA and p50/c-Rel heterodimers) in Bcl10-deficient B cells. Furthermore, a severe reduction in the formation of p52/RelB heterodimer was in agreement with reduced basal p100 protein levels combined with an inability to transcriptionally upregulate p100. Nonetheless, the limiting amounts of NF-κB2/p100 available in Bcl10-deficient B cells were efficiently processed into p52 and subsequently translocated into nucleus (Fig. 6B). Taken together, these data demonstrate that Bcl10 deficiency severely impairs the formation

FIGURE 5. Bcl10 deficiency impairs BAFF-induced canonical NF-κB activation. (A) Expression of BAFF-R. Splenocytes from wild-type and Bcl10-deficient mice were stained with Abs to CD19, IgM, IgD, and BAFF-R. Expression levels of BAFF-R on total splenic (CD19+), T1 (IgMhiIgD−), T2 (IgMhiIgDhi), and FO (IgMloIgDhi) B cells were measured by FACS analysis. (B–E) Splenic mature B cells (CD93+CD5−) were isolated from wild-type and Bcl10-deficient mice. (B) BAFF-induced association of Bcl10 with IKKβ. Wild-type or Bcl10-deficient mature B cells were stimulated with BAFF or anti-IgM for the indicated times. Cell lysates were immunoprecipitated with anti-Bcl10–conjugated Sepharose beads, followed by Western blotting analysis with the indicated Abs. (C) BAFF-induced IκBα phosphorylation. Cells were pretreated with MG132 and subsequently stimulated with BAFF for the indicated times. Cell lysates were subjected to direct Western blotting analysis with anti–phospho-IκBα or anti-actin Abs. (D and E) BAFF-induced canonical activation of NF-κB. Cells were stimulated with (+) or without (−) BAFF for 3 h and total cell lysates were subjected to NF-κB gel mobility shift (D) and Ab supershift (E) analysis. Arrows indicate the position of NF-κB complexes (D) and p50/p50 homodimers (E). Data are representative of three (A, C–E) or two (B) independent experiments.

FIGURE 6. Bcl10-deficient splenic B cells express reduced levels of p100, indirectly affecting BAFF-induced noncanonical NF-κB activation. (A) Expression of NF-κB/Rel family members. Protein levels of NF-κB/Rel family members in total cellular extracts from splenic mature B cells were determined by direct Western blotting analysis with the indicated Abs. Densitometric analysis of protein bands was performed and normalized to actin loading control. Levels of each NF-κB protein in Bcl10-deficient and wild-type B cells were compared by giving wild-type an arbitrary value of 1. The bar graph shows the relative p100 protein levels calculated from three independent experiments. p < 0.01. (B) BAFF-induced NF-κB2p100 processing and nuclear translocation. Following 16 h culture of splenic mature B cells with or without BAFF, cytoplasmic and nuclear extracts were prepared and subjected to Western blotting with the indicated Abs. GAPDH was used as cytoplasmic protein loading control and YY1 as nuclear protein loading control. (C) BAFF-induced NF-κB2p100 mRNA expression. Splenic immature (CD93+) and mature (CD93+CD23+) B cells were either stimulated with BAFF or left nonstimulated for 16 h. NF-κB2p100 mRNA expression was determined by quantitative real-time PCR. Relative fold induction in response to BAFF was normalized to 18S rRNA and calibrated to nonstimulated sample for the corresponding genotype. (D and E) BAFF-induced noncanonical activation of NF-κB. Splenic mature B cells were constantly stimulated with BAFF for 16 h and total cell lysates were subjected to NF-κB gel mobility shift (D) and Ab supershift (E) analysis. Arrows indicate the position of NF-κB complexes. (F) BAFF-induced ERK and Akt activation. Splenic mature B cells were stimulated with BAFF for the indicated times and cell lysates were subjected to Western blotting analysis with the indicated Abs. Data are representative of three (A, B, D, E) or two (F) independent experiments, or data are representative of at least three experiments that used one animal from each genotype per experiment (C).
of not only p50/RelA and p50/c-Rel complexes, typically activated by the canonical NF-κB pathway, but also p52/RelB complexes, typically activated by the noncanonical NF-κB pathway. The impairment of noncanonical NF-κB activation in Bcl10-deficient B cells is likely due to the reduction of NF-κB2/p100, which is in part regulated by the canonical NF-κB pathway.

In addition to the NF-κB pathway, BAFF stimulation promotes B cell survival by downregulating the proapoptotic molecule Bim via ERK activation as well as by activating the prosurvival kinase Akt (57, 58). To address the role that Bcl10 plays in these pathways, we examined whether Bcl10 is involved in BAFF-induced activation of ERK and Akt. Splenic mature B cells were isolated from wild-type and Bcl10-deficient mice and then stimulated with BAFF. The activation of ERK was evaluated by immunoblotting with Abs that detect phosphorylation of Thr202/Tyr204 within ERK1 and Thr185/Tyr187 within ERK2. BAFF-induced activation of ERK was comparable in Bcl10-deficient and wild-type B cells (Fig. 6F). Additionally, the activation of Akt was evaluated by immunoblotting with Abs that detect phosphorylation of Ser473 within Akt. BAFF-induced activation of Akt was biphasic. The initial activation, which occurred 5–10 min poststimulation, was reduced to basal level 30 min later, followed by a second phase of activation at 24 h (Fig. 6F). This biphasic activation of Akt by BAFF was not affected in Bcl10-deficient mature B cells (Fig. 6F). Thus, Bcl10 is not required for BAFF-induced activation of ERK and Akt.

**Bcl10 deficiency impairs BAFF-dependent NF-κB target gene expression**

The activation of the NF-κB pathway by BAFF stimulation ultimately upregulates the expression of antiapoptotic genes, such as Bcl-xL (22, 30). To confirm that Bcl10 plays an important role in BAFF-mediated survival of B cells through the NF-κB pathway, we examined BAFF-induced upregulation of the prosurvival NF-κB target gene, Bcl-xL, in Bcl10-deficient B cells. Following BAFF stimulation, the level of Bcl-xL proteins was markedly upregulated in wild-type B cells (Fig. 7). In contrast, the upregulation of Bcl-xL proteins was abolished in Bcl10-deficient B cells (Fig. 7). Note, the amount of Bcl-2 proteins remained unaltered before and after BAFF stimulation in both wild-type and Bcl10-deficient B cells (Fig. 7). Therefore, Bcl10 is required for BAFF-induced expression of Bcl-xL, a prosurvival gene of the Bcl-2 family that is regulated by NF-κB.

**Discussion**

Previous studies have demonstrated that the survival of all peripheral B cells as well as maintenance of anergic B cells depends on signals from both the BCR and BAFF-R (20–24). Our findings demonstrate that Bcl10 was essential for anergic B cell survival. This Bcl10 function was apparent in the diverse repertoire as well as in IgHEL single transgene. The Ig HEL single transgene did not appear to affect T1 and T2 B cells and only slightly reduced mature FO B cells in Bcl10-deficient relative to wild-type control mice, indicating that Bcl10 function was not critical for the survival of peripheral B cells in the absence of self-Ag. In IgHEL, sHEL double-transgenic model, soluble self-Ag, sHEL, drove self-reactive B cells into anergy in both wild-type and mutant mice. Anergic B cells that depend more heavily on BAFF underwent more apoptosis in the absence of Bcl10. Thus, the BAFF-R/Bcl10/NF-κB signaling axis contributes to the establishment of peripheral B cell tolerance by maintaining anergic B cells and preventing these B cells from self-Ag-induced elimination.

BAFF can induce canonical and noncanonical NF-κB activation pathways, both of which are required for B cell survival (35, 59). Whereas BAFF-R–induced activation of the noncanonical NF-κB pathway is relatively well studied, the membrane-proximal signaling cascades upstream of the canonical pathway remain unclear. Our current studies demonstrate that Bcl10 directly participated in BAFF-induced activation of canonical NF-κB via mechanisms involving direct association of Bcl10 with IKKβ. This result in the activation of the IKK complex consisting of IKKα, IKKβ, and NEMO. Our observation that noncanonical NF-κB activation by BAFF was impaired in Bcl10-deficient mice suggests a role for Bcl10 in the regulation of this NF-κB pathway as well. A direct role for Bcl10 in activation of the noncanonical pathway was ruled out by our data showing that Bcl10 deficiency did not affect NF-κB2/p100 to p52 processing. Instead, this defect was found to be an indirect effect of Bcl10 deficiency on the expression of NF-κB2/p100. Thus, our results suggest that reduced availability of NF-κB2/p100 limited the extent of noncanonical NF-κB activation in Bcl10-deficient B cells.

It is well known that Bcl10 forms a ternary complex with CARMA1 and MALT1 (CBM) to couple PKC activity to IKK complex activation during BCR-induced canonical NF-κB activation (48–51). Our data demonstrating a requirement for Bcl10 in the activation of the canonical pathway suggest that the CBM complex is required for BAFF-induced activation of the IKK complex. This hypothesis is supported by our finding that Bcl10 is constitutively associated with MALT1, suggesting that Bcl10/MALT1 partner with CARMA1 to form the CBM complex to activate the IKK complex during BAFF-R signaling. This possibility remains to be investigated, and the role of MALT1 in the activation of the canonical NF-κB pathway remains unknown. However,
a recent study has demonstrated that MALT1 regulates BAFF-induced noncanonical NF-κB activation in B cells. MALT1 deficiency impairs BAFF-induced phosphorylation and processing of NF-κB2/p100, as well as ReIB nuclear translocation, suggesting a direct role for MALT1 in the noncanonical NF-κB activation (60). Additionally, the role of CARMA1 in BAFF-induced canonical NF-κB activation is also unknown. Our findings suggest that the Bcl10-containing CBM complex mediates the canonical NF-κB pathway. Our results lay the foundation for further studies to determine the contribution of CARMA1 and MALT1 to BAFF-induced canonical NF-κB activation.

The membrane-proximal steps of the BCR signaling cascade that lead to the formation of the CBM complex have been well studied. Upon BCR ligation, protein tyrosine kinases Lyn, Syk, and Btk are sequentially activated, and B cell linker protein and CD19 are recruited to the receptor complex (61–64). The BCR complex subsequently activates PI3K and phospholipase C (PLC)γ2 (65, 66). Activated PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate (67, 68). In turn, diacylglycerol plus inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate–inhibited PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacygly