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The p85 β regulatory subunit of phosphoinositide 3-kinase has unique and redundant functions in B cells

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Abstract

Phosphoinositide kinase (PI3K) is activated by various receptors on lymphocytes and regulates development, activation, and tolerance. Genetic ablation of PI3K function in T cells leads to the appearance of autoimmune disorders. In B cells, loss of the class IA regulatory subunit p85 α causes a partial defect in B cell development and proliferation, whereas loss of p85 β alone causes no apparent changes in B cell function. Here we investigate further the consequences of p85 β deletion in B cells, in the presence or absence of p85 α . We demonstrate that p85 β partially compensates for loss of p85 α in B cell development and peripheral survival, with greater defects observed when both isoforms are absent. BCR-mediated AKT phosphorylation is partially reduced in p85 α -deficient B cells and further diminished with concomitant loss of p85 β . Unexpectedly, loss of p85 β results in increased BCR-mediated proliferation and ERK phosphorylation. These results indicate that the p85 β regulatory isoform has partially overlapping functions with p85 α in B cells as well as a unique role in opposing BCR responses.

Keywords

B cells; cell activation; cell proliferation; lymphocyte development; signal transduction

Introduction

Signaling through lymphocyte antigen receptors controls cell fate decisions during development and upon antigen recognition in the periphery. Antigen receptor signaling requires complex assemblies of signaling proteins and second messengers at the membrane, including the lipid products of PI3K enzymes [1,2]. Class I PI3Ks generate the phosphatidylinositol (PtdIns) derivatives PtdIns-3,4,5-P₃ and PtdIns-3,4-P₂, which recruit

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proteins containing a pleckstrin-homology domain to the cell membrane. Inhibitors of PI3K enzyme activity block lymphocyte proliferation [3–5]; however, the unique and overlapping functions of individual PI3K family members remain incompletely understood.

Class IA PI3Ks exist as a tightly associated heterodimer consisting of a catalytic subunit of approximately 110 kDa and a regulatory subunit [6,7]. The regulatory subunit isoforms have two SH2 domains that bind to the consensus sequence YXXM upon phosphorylation of the tyrosine residue, and this interaction increases activity and substrate access of the associated p110 catalytic subunit. Other protein:protein interactions involving modular domains within catalytic and regulatory subunits also regulate signaling responses [8–10]. There are three catalytic and five regulatory isoforms of class IA PI3K in mammals. In mice, *Pik3ca*, *Pik3cb*, and *Pik3cd* code for the catalytic isoforms p110 α , p110 β , and p110 δ , respectively. The *Pik3r1* gene encodes the regulatory isoforms p85 α , p55 α and p50 α through alternative promoter usage. The *Pik3r2* and *Pik3r3* genes encode p85 β and p55 γ , respectively. Gene targeting in mice has shown that PI3K activity in T cells must be properly regulated to maintain both antigen responsiveness and self-tolerance. Loss of p110 δ limits antigen-specific CD4 T cell expansion, but is also associated with reduced Treg function and mild colitis [11,12]. Similarly, loss of the regulatory subunits p85 α /p55 α /p50 α /p85 β diminishes proliferation but leads to development of lacrimal gland destruction resembling Sjögren's Syndrome [13,14].

PI3K is activated downstream of many receptors that mediate B cell responses including the antigen receptor (BCR), the CD19 coreceptor, toll-like receptors and cytokine receptors [1, 15]. Pharmacological and genetic studies have revealed that the class IA catalytic isoform p110 δ is crucial for B cell development, proliferation, and function [4,16]. The class IA regulatory subunit p85 α is also essential for normal B cell development and function; *Pik3r1*-null mice exhibit a partial block at the pre-B cell stage with an overall reduction in B cell populations in the spleen, bone marrow, and peritoneum, as well as a complete loss of BCR-mediated proliferation [3]. Similar defects occur in mice lacking only p85 α but retaining p55 α /p50 α (termed p85 α KO herein), which fits with the very low expression of the smaller isoforms in B cells [17–19].

Importantly, some mature B cells do develop in p85 α KO mice and these retain partial signaling and function [20–22]. The incomplete block in development and signaling in *Pik3r1*-null and p85 α KO mice suggests the possibility of functional compensation by other regulatory isoforms. Our initial analysis of *Pik3r2*-null (p85 β KO) mice revealed apparently normal B cell development and function [23]. To study further the redundant and unique functions of p85 α and p85 β , we generated conditional knockout mice in which B cells lack both *Pik3r1* and *Pik3r2*. We report that p85 β has a required function in B cell development and survival that is revealed upon concurrent loss of p85 α /p55 α /p50 α . In addition, we uncover a negative regulatory role for p85 β in BCR-mediated signaling and proliferation.

Results

Pik3r1 deletion is variable using CD19Cre but complete using CD21Cre

Assessing the phenotypes of B cells lacking both *Pik3r1* and *Pik3r2* required a conditional gene targeting approach, as combined deletion in all tissues causes embryonic lethality [24]. In order to create a B cell specific knockout of *Pik3r1* encoding p85 α /p55 α /p50 α , we bred *Pik3r1*-flox mice [25] with CD19-Cre mice in which the Cre recombinase is knocked into the CD19 locus [26]. Intercrosses with *Pik3r2*-null mice produced four genotypes: *Pik3r1*-flox (considered the wild-type (WT) control), *Pik3r1*-flox/*Pik3r2*-null (p85 β KO), *Pik3r1*-flox/CD19-Cre (referred to as BsKO₁₉, for B cell single knockout with CD19-Cre), and *Pik3r1*-flox/*Pik3r2*-null/CD19-Cre (BdKO₁₉, B cell double knockout with CD19-Cre). As previously seen in germline knockouts of *Pik3r1* or p85 α , BsKO₁₉ mice had a partial block in the proB/

preB transition in the bone marrow as well as reduced populations of mature subsets in the bone marrow, peritoneum, and spleen (see Supporting Information; Figure S1).

BdKO19 mice exhibited nearly identical developmental defects as BsKO19 mice. This does not rule out a role for p85 β in B cell development, since deletion of floxed alleles in CD19-Cre mice is not always efficient. Indeed, BdKO19 splenic B cells had comparable levels of p85 as wildtype (WT) and p85 $\beta^{-/-}$ (p85 β KO) B cells while BsKO19 B cells showed variable deletion (Figure S2). The reduced deletion efficiency in BdKO19 vs. BsKO19 supports the conclusion that the loss of p85 β together with p85 α /p55 α /p50 α produces a greater selective disadvantage during development than loss of p85 α /p55 α /p50 α alone. However, the variable deletion in BsKO19 mice complicated our attempts to further pinpoint crucial selection checkpoints.

In order to reduce the selective advantage of nondeleters during development, we crossed *Pik3r1*-flox/*Pik3r2*-null mice with CD21-Cre transgenic mice [27] to generate BsKO21 and BdKO21 strains. Driving Cre expression with CD21 regulatory elements limits *Pik3r1* deletion to late transitional and mature B cells, and follicular dendritic cells (FDC). Analysis of follicular B cells from the lymph nodes of BsKO21 and BdKO21 mice showed undetectable levels of p85 α (Figure 1A), confirming that deletion was efficient. Although the anti-pan-p85 antibody can detect p85 β in some cell types [3,23,24], no signal was observed in BsKO21 cells suggesting low p85 β expression in mature B cells. However, p85 β mRNA was readily detected in purified B cells from WT and BsKO21 mice but not in p85 β KO or BdKO21 (Figure 1B). Because class IA PI3Ks exist as a stable heterodimer, reduced regulatory subunit expression results in destabilization and downregulation of catalytic subunits [24,28]. Consequently, p110 α and p110 δ were undetectable and expression of p110 β was reduced in BsKO21 and BdKO21 B cells (Figure 1A).

Loss of p85 β exacerbates developmental and survival defects in the absence of p85 α

In accord with previous reports [20,23], follicular (FO) and marginal zone (MZ) B cell populations were reduced in BsKO21 but not in p85 β KO mice (Figure 1C, Supplementary Figure 3; Table I). Combined loss of p85 α and p85 β in BdKO21 mice resulted in greater decreases in the FO populations in the spleen, lymph nodes, and peritoneum compared to BsKO21 mice (Figure 1C, Supplementary Figure 3; Table I). MZ populations were similarly decreased in BsKO21 and BdKO21 mice, and the percentages and numbers of transitional subsets were unchanged. Peritoneal B1a populations, which do not express CD21, remained unaffected.

The defects seen in BsKO21 and BdKO21 mice suggested that there was decreased production or survival of mature B cells. In order to distinguish between these possibilities, we injected mice with BrdU for 24–72 h and analyzed BrdU incorporation in different B cell subsets by AA4.1/CD23/IgM staining (Figure 2A). Immature B cells from all four genotypes had comparable percentages of BrdU⁺ cells. This is consistent with the normal developmental profile of p85 β KO B cells and with CD21-Cre causing deletion of *Pik3r1* at a late transitional stage. However, BdKO21 mice had a higher percentage of BrdU incorporation in both FO and MZ B cell subsets after 72 h of treatment, whereas BsKO21 mice did not show any significant changes.

The absolute number of BrdU + mature B cells was not increased in BdKO21 (not shown), since there were fewer cells overall. One interpretation of these data is that BdKO21 mature B cells have decreased survival so that newly formed B cells represent a higher fraction of the mature B cell pool. To address this possibility, we carried out TUNEL staining of freshly isolated splenocytes, which revealed that BdKO21 B cells had an increased rate of apoptosis (Figure 2B). We then measured basal activity of the canonical NF κ B pathway, which is

required for maintenance of mature B cells [29]. The percentage of cells showing nuclear translocation of the p65 subunit of NF κ B1 was low and comparable among freshly isolated splenic mature B cells from WT, p85 β KO, BsKO21, and BdKO21 mice (Figure 2C).

B cells lacking p85 β exhibit an increased proliferative response to BCR cross-linking

To assess the functional defects in mature B cells, CFSE-labeled lymph node B cells from all four genotypes were stimulated with anti-IgM $\pm$ IL-4, anti-CD40 with IL-4, or LPS for 72 h (Figure 3A). In accord with previous studies of *Pik3r1* -null and p85 α KO B cells, BsKO21 B cells showed a complete block in response to anti-IgM that was only weakly restored by IL-4, yet proliferated normally in response to anti-CD40/IL-4. Surprisingly, BdKO21 B cells consistently showed low but detectable proliferation with anti-IgM treatment. The percentage of cells divided and the average number of divisions of the cycling cells (the division index) were significantly higher in BdKO21 compared to BsKO21 (Figure 3A, lower, and figure legend). Additionally, p85 β KO B cells from the lymph node also exhibited enhanced anti-IgM-induced proliferation compared to WT controls. Again, both the percent divided and the division indices were increased in p85 β KO vs. WT (Figure 3A, lower, and figure legend).

Proliferative responses to LPS (10 μ g/ml) were comparable among genotypes (Figure 3A), although at lower LPS concentrations the BsKO21 and BdKO21 showed slightly fewer divided cells (data not shown). The increased division in anti-IgM cultures was not due to significantly greater survival, as judged by propidium iodide exclusion at 48 h (Figure 3(B); compare p85 β KO vs. WT and BdKO21 vs. BsKO21) and Annexin-V + divided cells at 72 h (data not shown). We did observe an unexpected increase in survival of BdKO21 vs. BsKO21 B cells in culture medium alone (Figure 3B), contrary to the *in vivo* survival data (Figure 2B). This difference most likely results from distinct survival factors present in culture medium compared to intact lymphoid organs.

Altered BCR signaling in B cells lacking p85 isoforms

To investigate molecular mechanisms for altered function of B cells with different genotypes, we evaluated various signaling responses. Due to the low number of mature B cells that could be obtained from BdKO21 mice, we used the single cell methods of flow cytometry [22,30] or fluorescence microscopy. First we measured BCR-mediated Ca²⁺ mobilization. Generation of PtdIns(3,4,5)P₃ promotes optimal assembly of the BCR signalosome and PLC γ -mediated Ca²⁺ elevation [1,2]. Splenic B cells from p85 α KO cells display partially impaired Ca²⁺ flux whereas p110 δ KI B cells have similar defects as cells treated with the PI3K inhibitor LY294002 [4,22]. In order to determine whether p85 β contributes to BCR-mediated Ca²⁺ flux in the presence or absence of p85 α , we measured Ca²⁺ responses of immature, follicular, and MZ B cell subsets from WT, p85 β KO, BsKO21, and BdKO21 splenocytes (Figure 4A). Immature B cells from all four genotypes, which all retain *Pik3r1* at this stage, showed similar responses upon stimulation with anti-IgM.

This suggests that loss of p85 β does not impair Ca²⁺ signaling in transitional B cells. p85 β was also dispensable for Ca²⁺ mobilization in mature FO and MZ B cells; the responses of WT and p85 β KO were equivalent and BdKO21 cells did not display greater defects than BsKO21. Similar results were obtained from Ca²⁺ mobilization assays done using lymph node B cells (data not shown). The elevation of intracellular Ca²⁺ and diacylglycerol downstream of PLC γ initiates a pathway leading to nuclear entry of NF κ B transcription factors [1]. Thus, anti-IgM stimulation caused a large increase in the fraction of lymph node B cells exhibiting nuclear translocation of p65 (Figure 4B). Consistent with the Ca²⁺ data, the NF κ B response of p85 β KO cells was unaltered and BsKO21 and BdKO21 B cells had a similar reduction in NF κ B translocation. These results suggest that PI3K-dependent Ca²⁺ signaling and NF κ B activation are unique functions of p85 α with no redundant or compensatory role for p85 β .

The serine/threonine kinase AKT is a well-characterized downstream effector of PI3K that becomes activated upon recruitment to the cell membrane. p110 δ KI B cells have > 95% reduction in pAKT levels upon BCR stimulation whereas p85 α KO B cells show greater residual AKT phosphorylation [4,22]. In order to determine whether p85 β mediates the residual AKT activation, we stimulated lymph node B cells with anti-IgM and determined the level of phosphorylation of AKT on residue S473. The shift in fluorescence detection following BCR engagement was completely wortmannin-sensitive, demonstrating the specificity of detecting PI3K-dependent phosphorylation (Figure 5A; [30]). In accord with previous studies, p85 β KO B cells exhibited a normal response whereas BsKO21 B cells showed a partial defect similar to p85 α KO B cells. BdKO21 B cells displayed near complete abrogation of AKT phosphorylation, suggesting that p85 β partially compensates for the absence of p85 α in this arm of the PI3K signaling pathway. The AKT phosphorylation response of splenic FO B cells of different genotypes was comparable to the results from lymph node B cells (data not shown).

Activation of AKT triggers increases in metabolism and protein synthesis as cells prepare to undergo rapid rounds of cell division. These effects are partially due to indirect activation of the mammalian target of rapamycin (mTOR), a Ser/Thr kinase that phosphorylates proteins required for cell growth and protein synthesis such as 4E-BP1 and S6K [31,32]. In B cells, BCR-mediated mTOR activity is largely PI3K-dependent, as treatment with LY294002 or wortmannin blocks phosphorylation of S6K and its downstream effector S6 [22,33]. However, wildtype and p85 α KO B cells showed comparable S6 phosphorylation [22,33]. Similarly, both BsKO21 and BdKO21 B cells showed unimpaired S6 phosphorylation (pS6) upon stimulation with anti-IgM (Figure 5B). The direct mTOR inhibitor rapamycin blocked all pS6 detection, demonstrating specificity in this assay.

Inputs to S6 phosphorylation in BsKO21 and BdKO21 B cells were largely PI3K-independent, as indicated by wortmannin resistance of BCR-induced pS6 in these two genotypes. This suggests compensatory changes in the wiring of other signals affecting S6 kinases. We assessed the possible role of the RAF/MEK/ERK kinase cascade, which can promote S6K activity directly through phosphorylation of residues in the autoinhibitory domain, or through phosphorylation of TSC2 upstream of mTOR [32]. BsKO21 and BdKO21 B cells did not exhibit increased sensitivity to the MEK inhibitor U0126 (Figure 5B), indicating that this pathway does not play a major compensatory role in PI3K-independent S6 phosphorylation. Interestingly, however, BCR-mediated phosphorylation of ERK was greatly enhanced in BdKO21 and to a lesser extent in p85 β KO B cells (Figure 5C). This effect was consistently observed at 5 min post-BCR stimulation (Figure 5C) as well as 1 and 15 min (data not shown).

Discussion

Defining specificity in the function of related signaling proteins increases our understanding of lymphocyte biology and can reveal new molecular targets for modulating immune responses and autoimmunity. Applying this approach to the PI3K catalytic isoforms has defined crucial roles for the p110 δ (class IA) and p110 γ (class IB) enzymes in various aspects of innate and adaptive immunity [34–36]. Our previous work showed that p85 β has a unique negative regulatory role in TCR survival, together with a positive role in T cell proliferation that is redundant with other regulatory isoforms [13,23]. Here, we have carried out a detailed comparison of the shared and redundant functions of p85 α and p85 β in B cell development, proliferation and signaling. Our results confirm that p85 α has a B cell intrinsic function as the predominant class IA regulatory isoform for many responses, while revealing previously unknown roles for p85 β .

Comparison of BsKO19 and BdKO19 mice suggests a strong selection pressure to maintain PI3K signaling during development. Although BdKO19 B cells do not have a more severe

developmental defect than BsKO19 B cells they also have lower deletion efficiency, which suggests that loss of p85 β further decreases fitness in the absence of p85 α . One possible mechanism could be decreased survival mediated by c-Kit and other cytokine receptors during B cell development in the bone marrow. In mast cells, loss of p85 α or p85 α /p55 α /p50 α causes only partially impaired responses to c-Kit ligand (stem cell factor) [37,38]. The cytoplasmic tail of c-Kit contains two tyrosines that when phosphorylated can recruit class IA PI3K regulatory subunits, with p85 β possibly able to compensate in the absence of p85 α .

Using the CD21Cre system we observed that deleting p85 α /p55 α /p50 α reduces mature B cell numbers without increasing turnover or apoptosis *in vivo*. This supports our previous finding that the primary role of p85 α is to promote developmental progression to mature FO and MZ subsets, rather than to maintain survival [20]. Deleting p85 α /p55 α /p50 α together with p85 β further diminishes mature B cell numbers while also increasing turnover and apoptosis. This suggests that *Pik3r1* and *Pik3r2* gene products have shared but required functions in mature B cell maintenance. The decreased survival might result from impaired tonic BCR signaling and/or reduced responsiveness to BAFF [27,39], or to a defect in the FDC populations of BdkO21 mice.

Do different PI3K isoforms merely exist for compensatory reasons or do they have distinct functions? One interpretation is that regulatory isoforms are redundant and that distinct knockout phenotypes reflect differences in expression levels. However, p85 β has unique functions in T cells, which like B cells have low expression of p85 β relative to p85 α . p85 β KO but not p85 α KO T cells have increased survival and proliferation *in vitro* [19,23]. There is also a greater reduction in basal motility *in vivo* of p85 β KO T cells compared to p85 α KO [40]. Our current results indicate that both p85 α and p85 β have unique functions in BCR-mediated signaling and function. p85 α is required for maximal BCR-stimulated Ca²⁺ mobilization, whereas p85 β appears dispensable for this response even in the absence of p85 α .

A similar pattern was observed for NF κ B activation, known to be downstream of the BCR/Ca²⁺ signalosome. Unexpectedly, we find that p85 α and p85 β have opposing roles in BCR-mediated proliferation. The response is eliminated in BsKO21 B cells, and slightly but reproducibly increased in p85 β KO relative to WT B cells. A negative regulatory role for p85 β in BCR responses is further supported by the partial restoration of proliferation in BdkO21 vs. BsKO21 B cells, in cultures treated with anti-IgM.

The increased response of p85 β -deficient cells did not result from greater NF κ B activation but was associated with enhanced ERK phosphorylation. Studies of p85 α KO B cells have shown a similar enhancement of BCR-induced ERK phosphorylation [21,22]; BsKO21 cells showed a similar trend here but with greater variability. It has been shown that pan-PI3K inhibitors or p110 δ -specific inhibitors partially decrease ERK phosphorylation in B cells [4,22,41]. The opposing actions of PI3K catalytic and regulatory subunits on ERK phosphorylation add to the accumulating evidence that the regulatory subunits of PI3K have functions that are independent of their ability to bind the catalytic subunits [9,42,43]. Further experiments are necessary to uncover the mechanism of enhanced ERK phosphorylation.

The increased proliferation of p85 β KO lymph node (FO) B cells *in vitro* was quantitatively modest, and not detected by thymidine incorporation using unfractionated splenic B cells [23]. However, the small increase in FO B cell responsiveness might have important biological consequences. When we analyzed older p85 β KO mice, we observed many with mild lymphocyte infiltrates in the salivary gland, as well as anti-nuclear antibodies in ~ 15% of mice between 3 and 12 months of age [14]. We also detected ~ 30% higher basal serum IgM levels in young adult p85 β KO mice ($p < 0.05$). In *Pik3r2*-null mice that also have T cell-specific deletion of *Pik3r1*, a severe autoimmune exocrinopathy develops with lacrimal gland

destruction and high titers of anti-nuclear antibodies [14]. It is likely that loss of p85 β in a non-T cell is required for disease development, as only subclinical infiltrates are observed when *Pik3r2* is intact and *Pik3r1* is deleted in T cells (J.S.O., unpublished observations). Hence, mild hyperreactivity of p85 β KO B cells might be an important contributing factor to this complex autoimmune syndrome. Human autoimmune diseases are usually associated with complex combinations of genetic risk factors, so it is possible that altered p85 β expression or function contributes to antibody-mediated autoimmune pathology.

It is intriguing that p85 β could partially compensate for p85 α in BCR-triggered AKT activation but not in Ca²⁺ mobilization. This suggests the existence of distinct pools of 3'-phosphorylated inositol lipids that recruit AKT or PLC γ downstream of different signaling complexes. The complex that promotes AKT recruitment and activation can function with either p85 α or p85 β , whereas the Ca²⁺ signalosome is selective for p85 α . We speculate that redundant recruitment occurs when assembly is based mainly on the highly conserved SH2 domains, whereas unique recruitment occurs when assembly requires SH3 or RhoGAP-homology domains that are less similar in p85 α and p85 β .

In summary, our results demonstrate that p85 α and p85 β have overlapping roles in B cell development and peripheral survival, and that p85 β acts in a pathway that dampens ERK activation and proliferation following BCR crosslinking. Considering that mice lacking either p85 α or p85 β are viable [19,23,44], it might be possible to design therapies that modify expression or function of these regulatory isoforms to treat immune diseases without causing severe toxicity.

Materials and methods

Mice

The generation of *Pik3r1*-flox and *Pik3r2*-null mice is described elsewhere [25,44]. To generate the various conditional knockouts described here, *Pik3r1*-flox and *Pik3r2*-null mice were intercrossed and then bred to CD19-Cre or CD21-Cre transgenic mice. Mice were maintained in a C57BL/6 $\times$ 129SvEv mixed background and used at 6 to 16 weeks of age. Animals were housed under sterile conditions with irradiated food and autoclaved microisolator cages and water. All procedures were approved by the institutional animal care and use committee of UC Irvine.

FACS analysis

Single cell suspensions of RBC-depleted spleens, lymph nodes, and peritoneal lavages were stained with combinations of the following antibodies: anti-CD5-FITC, anti-CD21-FITC, anti-CD24-PE, anti-CD23-biotin, anti-AA4.1-PE, anti-Thy1.2 PE (eBiosciences, San Diego, CA), anti-CD43-PE, anti-IgM-PerCP, anti-B220-APC (BD Biosciences, San Diego, CA), anti-BrdU-FITC, and Annexin V- PE (Caltag, Burlingame, CA). Samples were acquired on a FACScalibur (BD Biosciences) and data analyzed using FlowJo (Tree Star, Inc., San Carlos, CA) software.

B cell purification and culture

B cells were purified via negative selection using anti-CD43 microbeads on MACS columns (Miltenyi, Auburn, CA), as described [33]. For removal of immature B cells, cells were also incubated with anti-AA4.1-biotin and anti-biotin microbeads. Purity was verified to be 85–95% by FACS analysis. Cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FCS, 5 mM HEPES, 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 μ g/ml), and 50 μ M β -mercaptoethanol for all assays shown.

Immunoblotting

Purified lymph node B cells (2 or 3×10^6) were lysed in a detergent buffer containing 1% TritonX-100, 50mMTris, pH 7.4, 10% glycerol, 150 mM NaCl and a protease inhibitor cocktail (Sigma, St. Louis, MO). Proteins were resolved via 7.5% or 12.5% SDS-PAGE (Biorad, Hercules, CA) and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk in TBST for 1 hr, washed with TBST, and probed with primary antibodies for 1 hr at room temperature (RT). Antibodies against p110 α , p110 β , p110 δ (Santa Cruz, Santa Cruz, CA), and a rabbit antiserum that recognizes all class IA PI3K regulatory isoforms (anti-pan-p85, kind gift from Lewis Cantley) were used to probe for p85 α and p85 β expression. mAbs specific for β -Actin (Sigma, St. Louis, MO) were used as loading controls.

RNA expression analysis

Total cellular RNA was extracted by using TRIzol reagent (Invitrogen Life Technologies) and DNase-treated. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). The p85 β primers were designed to detect exons deleted by the gene targeting strategy. Primer sequences were as follows. p85 β forward: TTCCAGTACAGGGCTGTGTACCCATT; reverse: TAGCATTCAGTGTCCAGCTCTGCT; GAPDH forward: AATTCACGGCACAGTCA; reverse: GCGGCACGTCAGATCCACGACGGAC. PCR was done for 30 cycles as follows: 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, with a 7-min final extension at 72°C.

B cell proliferation

B cells from the spleen or lymph nodes were purified as above. Cells were stimulated with 10 μ g/mL F(ab')₂ fragment of anti-IgM (Jackson ImmunoResearch, West Grove, PA), 10 μ g/mL LPS (Sigma), or 1 μ g/mL anti-CD40 (BD Biosciences) and 2 ng/mL IL-4 (R&D) for 72 hrs. Cell division and death were tracked following CFSE dilution and Annexin-V staining, respectively.

BrdU incorporation

Mice were injected intraperitoneally with 1200 μ L of 3 mg/mL BrdU in PBS every 12 h for the times described. Splenocytes were then harvested, stained with anti-CD23-PE, anti-IgM PerCP, and anti-AA4.1-biotin/SA-APC, and stained for BrdU incorporation using the BrdU Flow Kit (BD Biosciences).

Survival assays

Freshly isolated splenocytes were TUNEL stained using the In Situ Cell Death Detection Kit, fluorescein (Roche, Indianapolis, IN). Purified mature B cells were incubated in medium alone or stimulated with 20 μ g/mL anti-IgM F(ab')₂ (Jackson Immunoresearch). Cells were stained with 2 mg/ml propidium iodide (Invitrogen, Carlsbad, CA) and incubated for 5 min at RT prior to analysis.

Calcium flux assay

Splenocytes were stained with CD1d-biotin/SA APC and CD24-PE prior to loading with the calcium indicator dyes Fluo-3 and Fura Red (Invitrogen), as described previously [30]. The baseline level of Fluo-3/Fura Red was collected for 1 min before cells were stimulated with 10 μ g/mL anti-IgM for an additional 10 min.

NF κ B translocation assay

B cells were initially stimulated for 5 min in a 37°C water bath then allowed to settle onto polylysine coated coverslips for an additional 55 min in a 37°C incubator. Cells were fixed with 1

mL 4% paraformaldehyde for 10 min, washed with PBS, then stained with anti-p65 (Santa Cruz) in 0.1% Triton-X/PBS for 1 hr at RT, washed, then stained with anti-rabbit IgG Alexa 488 (Invitrogen) for 15 min at 4°C. The coverslips were mounted using Vectashield with DAPI (Vector Laboratories, Burlingame, CA), sealed, then visualized with a Zeiss Axiovert microscope equipped with an Axiocam CCD camera. Digital images were scored for percent translocation by an observer who was blinded to the sample description.

Phosflow assays for pAKT, pERK, pS6

Lymph node B cells incubated for 20 min at 37°C in media alone or in the presence of 50 nM wortmannin, 10 ng/mL rapamycin, or 10 mM U0126 (EMD, San Diego, CA) prior to stimulation. Cells were stimulated with 10 µg/mL F(ab')₂ fragment of anti-IgM for 5 min (pAKT, pERK) or 15 min (pS6) and stained intracellularly as described [28]. Briefly, cells were fixed in 2% MeOH-free paraformaldehyde (Polysciences Inc., Warrington, PA), pelleted, and permeabilized with 90% MeOH for 30 min on ice. Cells were then washed, blocked with 0.5% BSA in PBS, then stained with anti-pAKT (S473), anti-pERK (T202/Y204), or anti-pS6 (S235/S236) (Cell Signaling Technology, Danvers, MA) for 1 hr. pAKT and pERK samples were additionally stained with donkey anti-rabbit biotin (Jackson ImmunoResearch) for 30 min. Cells were then stained with B220-FITC, Thy1.2-PE, and anti-rabbit IgG Alexa 647 (pS6) or SA-APC (pAKT, pERK).

Statistical analyses

To test for significant differences, data were analyzed using either Student's t-tests or one-way ANOVA and Tukey post-test, as noted in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PI3K	phosphoinositide 3-kinase
PtdIns	phosphatidylinositol
mTOR	mammalian target of rapamycin
WT	wild-type
KO	knockout

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Figure 1.

Efficient deletion and development defects in BdKO21 mice, (A) Immunoblot analysis of purified lymph node B cells from WT, p85βKO, BsKO21, and BdKO21 mice reveals the absence of p85α and downregulation of catalytic subunits in BsKO21 and BdKO21 B cells. The absence of a detectable p85 band in BsKO21 indicates that p85β is not greatly upregulated. Other than the anti-pan-p85 antiserum used here, no other antibodies are available that detect p85β selectively and sensitively in murine cells. (B) RT-PCR analysis of RNA from purified lymph node B cells confirms that the *Pik3r2* gene encoding p85β is transcribed in FO B cells. (C) Quantitation of FACS data from multiple mice (example in Figure S3) shows that BsKO21 and BdKO21 mice have graded defect in mature B cell subsets in the spleen and periphery. $p < 0.05$, $^{**}p < 0.001$ vs. WT; $^{†}p < 0.05$ BsKO21 vs. BdKO21, using one-way ANOVA.

Figure 2.

(A) BdKO21 mice have impaired mature B cell maintenance. Mice were injected i.p. with 600 μ g of BrdU every 12 h for 24, 48, or 72h. Splenocytes were then harvested, surface stained with anti-CD23/AA4.1/IgM, and analyzed for BrdU incorporation. The graphs show the averages $\pm$ SEM with three or four mice per condition. * p < 0.05 using one-way ANOVA (B) Scatter plot diagram showing the percentage of TUNEL+ cells B220+ and B220- populations in freshly isolated splenocytes. Each point represents an individual mouse. * p < 0.05 (C) Splenic mature B cells were stained for p65 subunit of NF κ B1 and DAPI to determine the percentage showing nuclear localization of NF κ B by immunofluorescence. Data are an average of three different experiments $\pm$ SEM where at least 100 cells were counted per point by a blinded observer. * p < 0.05.

Figure 3.

(A) Loss of p85 β results in increased proliferation upon BCR stimulation. Lymph node B cells from WT, p85 β KO, BsKO21, and BdKO21 mice were stimulated with the indicated mitogens for 72 h. Division was determined by CFSE dilution, gating on live cells among 40,000 total events. Data are representative of three experiments. Quantitative analysis of the CFSE dilution data revealed that loss of p85 β leads to a higher division index upon anti-IgM treatment (lower panel; mean $\pm$ SEM, $n = 3$). The percentages of live, divided cells among different genotypes were 13.8 ± 1.1 (WT), 26.8 ± 3.6 (p85 β KO), 0.002 ± 0.001 (BsKO21) and 2.4 ± 0.3 (BdKO21). * $p < 0.05$ compared to WT, *** $p < 0.001$ vs. WT, † $p < 0.001$ vs. BsKO21 (B) Splenic mature B cells were cultured in media alone or with 20 μ g/mL anti-IgM and analyzed for cell viability by PI exclusion at the time points indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. WT; † $p < 0.05$ BsKO21 vs. BdKO21, using one-way ANOVA.

Figure 4.

(A) p85 α mediates calcium flux upon BCR crosslinking. Splenocytes were stained with CD1d and CD24 to differentiate immature, FO, and MZ populations. Calcium influx defect in BsKO21 and BdKO21 B cells is seen in the FO and MZ populations (which lack p85 α) but not in immature B cells (which still express p85 α). (B) NF κ B activation in LN B cells was determined by p65 translocation to the nucleus. Data shown is the average of three experiments $\pm$ SEM. * $p < 0.05$, using one-way ANOVA.

Figure 5.

Differential functions of p85β in BCR-mediated phosphorylation of AKT and ERK. Lymph node cells were stimulated with 10 μg/mL anti-IgM to measure levels of pAKT (A), pS6 (B), and pERK (C). Cells were pretreated with 50 nM wortmannin, 10 ng/mL rapamycin, or 10 μM U0126 for 15 min where indicated. Left panels show representative histogram overlays of three to five independent experiments. In panel B, left, the U0126 histograms are omitted for clarity. Right panels show the averages of normalized mean fluorescence intensity (MFI) values ± SEM. MFIs of each experiment were normalized using the value of the unstimulated WT cells. * $p < 0.05$, ** $p < 0.01$ vs. WT; † $p < 0.05$ BsKO21 vs BdKO21 with Student's paired two-tailed t test.

Table I

Total numbers of splenic B cell subsets.

	B cell subsets ($\times 10^6$ cells) (mean $\pm$ SEM)			
	T1	T2	FO	MZ
WT	10.8 $\pm$ 2.1	7.4 $\pm$ 2.1	27.6 $\pm$ 2.6	4.8 $\pm$ 0.5
P85 β KO	7.3 $\pm$ 0.8	6.1 $\pm$ 0.9	26.0 $\pm$ 2.0	3.9 $\pm$ 0.7
BsKO21	15.3 $\pm$ 3.4	7.0 $\pm$ 2.3	18.3 $\pm$ 2.0 [★]	1.3 $\pm$ 0.2 ^{★★}
BdKO21	9.2 $\pm$ 1.7	4.1 $\pm$ 1.3	10.3 $\pm$ 1.0 ^{†#}	1.6 $\pm$ 0.7 [★]

[★]
 $p < 0.05$,^{★★}
 $p < 0.01$,[†]
 $p < 0.001$ vs. WT,[#]
 $p < 0.05$ vs. BsKO21, using one-way ANOVA.