Functional phenotype of phosphoinositide 3–kinase p85α–null platelets characterized by an impaired response to GP VI stimulation

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Phosphoinositide 3–kinases (PI3Ks), a family of lipid kinases comprising 3 classes with multiple isoforms, have been shown to participate in different phases of platelet signaling. To investigate the roles that enzymes play in platelet function in vivo and determine which isoforms are important for particular signaling events, we analyzed platelet function of gene knockout mice deficient in the p85α regulatory subunit of heterodimeric class IA PI3K. The kinase activity of p85α−/− platelets was only 5% of the activity of platelets from wild-type littermates. Platelet aggregation induced by adenosine diphosphate (ADP), thrombin, U46619, phorbol 12-myristate 13-acetate (PMA), or botrocetin was not defective in p85α−/− mice, compared with wild-type animals. In contrast, aggregation induced by collagen and collagen-related peptide (CRP) was partially but readily impaired in p85α−/− mice. Both P-selectin expression and fibrinogen binding in response to CRP were also decreased to a similar extent in p85α−/− platelets. Platelets from p85α−/− mice appeared to spread poorly over a CRP-coated surface with intact filopodial protrusions. Significant attenuation of CRP-induced tyrosine phosphorylation in known PI3K effectors such as Btk, Tec, PKB/Akt, and phospholipase Cγ2 were observed with p85α−/− platelets, whereas no alteration was noted in upstream molecules of Syk, LAT, and SLP-76. Considered as a whole, these results provide the first genetic evidence that PI3K p85α plays a significant role in platelet function, almost exclusively in the glycoprotein (GP) VI/Fc receptor γ chain complex–mediated signaling pathway. (Blood. 2003;102:541-548)

Introduction

Phosphoinositide 3–kinases (PI3Ks) constitute a family of lipid kinases that are ubiquitously expressed in many cell types. These enzymes play a key role in the regulation of a variety of cellular processes: proliferation, survival, glucose metabolism, cytoskeletal remodeling, and vesicular trafficking.[1] PI3Ks phosphorylate an inositol ring of membrane-embedded inositol phospholipid at the 3′ position and generate D3 phosphoinositides (PtdIns-3-P; PtdIns-3,4-P2; PtdIns-3.5-P2; and PtdIns-3,4,5-P3), which then function as potent second messengers to relay signals by recruiting downstream molecules to the vicinity of the cellular membrane.[2,3] The most favored targets of the enzyme via its phosphoinositide products are the pleckstrin homology (PH) domain–containing effector molecules. These include Tec family tyrosine kinases, serine/threonine kinases such as Akt/protein kinase B (PKB), guanosine diphosphate/guanosine triphosphate (GDP/GTP) exchange factor (GEF) families such as Vav, and phospholipase (PLC)γ subtypes.[1-3] Of the 3 classes of PI3K (classes I-III), class IA and IB enzymes have been extensively studied in platelets[4-6] and are positioned downstream of membrane receptor stimulation and preferentially catalyze phosphorylation of PtdIns-4,5-P2 in vivo. The class IA subclass enzyme is a heterodimer comprising p110 catalytic and regulatory subunits, and to date 3 p110 catalytic subunits (α, β, and δ) and 3 regulatory subunits (85α, 85β, and 55γ) derived from different genes have been reported.[1] In addition, the 85α protein possesses 2 alternatively spliced variants, p55α and p50α.[7] Regulatory subunits act as adaptor molecules to activate the p110 catalytic subunit via interactions between their Src homology 2 (SH2) domains and specific phosphorylated tyrosine residues (Y-X-X-M motif) of upstream signaling molecules. In addition, class IA enzymes are known to be activated by G proteins.[8,9] A class IB subclass enzyme, PI3Kγ, is also abundant in platelets; it comprises a heterodimer complex with a p110γ catalytic subunit and a unique p101 regulatory subunit. The enzyme has been shown to be specific for G protein–coupled receptor (GPCR) activation through G protein βγ subunits.[4,10] Class IA PI3K utilizes a series of nonreceptor tyrosine kinases and associated adaptor molecules when it is activated downstream of adhesion receptor engagement to transduce signals. In fact, the collagen-induced platelet

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activation pathway via the glycoprotein (GP) VI/Fc receptor γ chain (FcRγ) complex evokes significant lipid kinase activity.[11] Upon GP VI cross-linking by collagen, immunoreceptor tyrosine-based activation motifs (ITAMs) displayed on the FcRγ subunit are phosphorylated by the protein tyrosine kinases Lyn and Fyn, leading to binding and activation of the Syk tyrosine kinase. Following assembly with adaptor molecules such as LAT and SLP-76, PI3K is activated via its p85 adaptor subunit.[12,13] With GP VI stimulation, platelet activation induced by aggregated immunoglobulin G (IgG) also involves class IA PI3K as a major signaling element associated with another ITAM-containing receptor, FcγR IIA.[14,15] Class IA enzymes are also implicated in processes downstream of GP Ib/IX/V complex–mediated signaling[16] and upstream of integrin α_{IIb}/β_3 (GP IIb/IIIa) complex activation (inside-out signaling),[4] and in postintegrin cellular responses (outside-in signaling), including conformational changes induced by actin rearrangement.[17,18] Platelet activation stimulated by GPCRs is physiologically important for platelet thrombus formation and is closely associated with PI3K activity.[4] Since PI3Kγ is activated only by G protein βγ subunits, the enzyme may be responsible for causing the observed increase in PI3K lipid products in response to stimulation with thrombin, adenosine diphosphate (ADP), and the thromboxane A_2 analog U46619.[4,19,20] However, class IA PI3K is also activated by GPCRs, and this type of activation may involve either a nonreceptor tyrosine-phosphorylated intermediate or the direct engagement of G protein βγ subunits.[9,21] Although a large body of evidence has clearly demonstrated that PI3K is intimately associated with different phases of platelet activation, the exact roles the enzyme plays in platelet functions in vivo and which isoforms are important for particular signaling events are yet to be determined. Platelets are terminally differentiated anucleate cells, and conclusions from most studies predominantly rely on indirect measurement of PI3K activity using structurally distinct inhibitors of PI3K (wortmannin and LY294002). This means that data analyses may be inherently limited in terms of specificity. To directly address these issues, we analyzed the function of platelets deficient in class IA PI3K p85α proteins in gene knockout mice.

Materials and methods

Materials and antibodies

Adenosine 5′-triphosphate (ATP), apyrase, phorbol 12-myristate 13-acetate (PMA), Arg-Gly-Asp-Ser (RGDS) peptide, acetylsalicylic acid (ASA), prostaglandin E_1 (PGE_1), bovine serum albumin (BSA), and poly-L-lysine were all purchased from Sigma-Aldrich (Tokyo, Japan). Phosphatidylinositol was obtained from Funakoshi (Tokyo, Japan). U46619 and A23187 from Merck KGaA (Darmstadt, Germany), and adenosine diphosphate (ADP) from Biopool (Ventura, CA). Collagen was supplied by Nycomed (Munchen, Germany). Collagen-related peptide (CRP) was prepared as previously described.[22] Botrocetin was a generous gift from Dr Yoshihiro Fujimura (Nara Medical College, Kashiwara, Japan). Human thrombin was provided by Welfide (Osaka, Japan). Alexa Fluor 488–conjugated human fibrinogen was from Molecular Probes (Eugene, OR). Fluorescein isothiocyanate (FITC)–conjugated antimouse P-selectin antibody was purchased from BD Pharmingen (San Diego, CA). Polyclonal anti-Btk antibody was kindly provided by Dr Owen Witte (University of California, Los Angeles). Anti-Syk, PLCγ2, SLP-76, p110α, p110β, p110δ and Akt/PKB polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antiphosphotyrosine monoclonal antibody (clone 4G10) and anti-PI3K $p85^{PAN}$, LAT, and Tec polyclonal antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Anti–phospho-Akt antibody was obtained from New England Biolabs (Beverly, MA). A specific antibody against p85β was prepared as described.[23] Thin-layer chromatography (TLC) plates were purchased from Whatman International (Maidstone, United Kingdom).

Mice

PI3K p85α −/− mice[24,25] were backcrossed to C57BL/6 mice for more than 7 generations before intercrossing heterozygous mice. The targeting strategy allowed selective disruption of p85α expression, while leaving gene products for p55α and p50α isoforms intact.[25] All mice were maintained under strict pathogen-free conditions. All experiments were performed in accordance with Keio University Institutional Guidelines.

Bleeding time

At 2 to 3 months of age, mice were anesthetized using diethylether. An incision was made 1 cm from the tip of the tail, and the emerging blood was blotted onto Whatman 3M paper (Whatman International) every 15 seconds. Bleeding times were defined as the time required for all visible signs of bleeding to stop.[26]

Blood collection and preparation of platelets

Whole murine blood was collected in syringes containing 100 μL acid citrate dextrose (ACD; 120 mM sodium citrate, 110 mM glucose, and 80 mM citric acid) by cardiac puncture under diethylether anesthesia. Blood cell counts were determined using an automated blood cell counter. Blood from 2 to 6 mice (2 to 4 months old, both sexes) was pooled for preparing platelet samples. Platelet-rich plasma (PRP) was obtained by centrifuging whole blood at 660*g* for 1 minute at 22°C. The residual blood sample was diluted using 400 μL RCD solution (36 mM citric acid, 5 mM glucose, 5 mM KCl, 103 mM NaCl, and 2 μM PGE_1, pH 6.5) containing 10% (volume/volume) ACD and 0.4 U/mL apyrase, then centrifuged at 660*g* for 1 minute to recover diluted PRP. To prepare washed platelets, PRP was combined with diluted PRP pretreated with 0.2 mM ASA at 37°C for 15 minutes and diluted using an equal volume of RCD solution containing 10% (vol/vol) ACD and 0.4 U/mL apyrase. Washed platelets were then obtained by centrifuging at 1000*g* for 7 minutes. Isolated platelets were resuspended at a final concentration of 5×10^5/μL in modified Tyrode-HEPES buffer (134 mM NaCl, 0.34 mM NaH_2PO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid], and 5 mM glucose, pH 7.3) containing apyrase.

Aggregation studies

Platelet concentrations of PRP were adjusted to 3×10^5/μL using platelet-poor plasma (PPP). A total of 125 μL PRP was placed in siliconized glass tubes and incubated at 37°C for 10 minutes before stimulation. Aggregation was optically monitored using a platelet aggregometer (Hema Tracer TM Model 601; Niko Bioscience, Tokyo, Japan). PPP was used as a reference to indicate 100% aggregation. For studies utilizing thrombin, washed platelets without pretreatment under ASA were suspended in modified Tyrode-HEPES buffer before use.

Flow cytometry

Washed platelets suspended in modified Tyrode-HEPES buffer containing 0.4 U/mL apyrase were labeled using FITC-conjugated antimouse P-selectin antibody (with 1 mM RGDS peptide and 1 mM $CaCl_2$) or Alexa Fluor 488–conjugated human fibrinogen (1 mM $CaCl_2$). Following stimulation with the appropriate concentrations of CRP, labeling of murine platelets was performed for 30 minutes. Samples were then analyzed using a FACSCalibur flow cytometer (Nippon Becton Dickinson, Tokyo, Japan).

Morphologic analysis of adherent platelets under electron microscopy

Control discoid platelets were obtained as follows: platelets in modified Tyrode-HEPES buffer containing 2 mM $MgCl_2$ were fixed in 1% glutaraldehyde, placed on poly-L-lysine–coated coverslips, and allowed to adhere to the surface for 60 minutes. Collagen and CRP were diluted to 10 μg/mL and

6 μg/mL, respectively, with modified Tyrode-HEPES buffer and immobilized on coverslips in culture dishes overnight at 4°C. Coverslips were washed 3 times with modified Tyrode-HEPES buffer and incubated with 2% BSA for 2 hours at room temperature for blocking. Platelets suspended in modified Tyrode-HEPES buffer containing 2 mM $MgCl_2$ (1×10^5 platelets/μL, 100 μL) were brought into contact with collagen- or CRP-coated coverslips in culture dishes and platelets were allowed to adhere at 37°C for 10 minutes. After rinsing 3 times to remove nonadherent platelets, coverslips were further incubated at 37°C for 20, 50, and 80 minutes. Adherent platelets were fixed using 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 minutes. Fixed platelets adhering to coverslips were washed 5 times in 0.1 M phosphate buffer (pH 7.4), postfixed using 1% osmium tetroxide in the same buffer for 15 minutes, dehydrated in a graded ethanol series, and then dried using a Hitachi ES-2020 freeze dryer (Hitachi, Tokyo, Japan) and *t*-butyl alcohol. Specimens were coated with an amorphous and continuous layer of sublimated osmium tetroxide (approximately 10 nm) using an NL-OPC80 osmium plasma coater (Nippon Laser & Electronics Lab, Nagoya, Japan). Slides were then examined under a Hitachi S-4500 field emission scanning electron microscope (Hitachi) at an accelerating voltage of 10 kV. Lengths of filopodia, defined as protrusions from the platelet body less than 130 nm in width, were measured using NIH Image software (National Institutes of Health; http://rsb.info.nih.gov/nih-image/). The area of the CRP-coated slides covered by platelets was analyzed using the same software.

Immunoprecipitation and Western blotting

Platelets in modified Tyrode-HEPES buffer containing 0.4 U/mL apyrase, 1 mM RGDS peptide, and 1 mM EGTA (ethylene glycol tetraacetic acid) were stimulated using the appropriate concentrations of collagen or CRP. Reactions were terminated with the addition of an equal volume of ice-cold lysis buffer (2% Nonidet P40 [NP-40], 20 mM Tris [tris(hydroxymethyl)aminomethane], 300 mM NaCl, 10 mM EDTA [ethylenediaminetetraacetic acid], 2 mM Na_3VO_4, 10 mg/mL aprotinin, 1 mg/mL pepstatin, 10 mg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.3). Debris was removed by centrifugation at 20 000*g* for 10 minutes. Platelet lysates were incubated with antibodies for various proteins and protein A–sepharose beads (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) for 2 hours at 4°C under continuous rotation. The beads were washed extensively in 2-fold diluted lysis buffer. Precipitated proteins were extracted in Laemmli sample buffer and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Separated proteins were transferred to Hybond-ECL membrane (Amersham Pharmacia), which was then blocked in 10% BSA in TBS-T (20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20) and hybridized sequentially using primary antibodies and horseradish peroxidase–conjugated secondary antibody (Amersham Pharmacia). Bound antibodies were detected using an enhanced chemiluminescence (ECL) Western blotting kit (Amersham Pharmacia).

PI3K assay

Platelets were lysed and immunoprecipitated using anti-$p85^{PAN}$ antibody (Upstate Biotechnology), which reacts with all 3 p85α isoforms (p85α, p55α, and p50α) in addition to p85β and p55γ subunits. Beads were washed 3 times in PI3K buffer (25 mM Tris, 0.5 mM EGTA, and 100 mM NaCl, pH 7.4) and suspended in PI3K buffer containing 200 μg/mL phosphatidylinositol. Following preincubation at 37°C for 10 minutes, the reaction was initiated with the addition of 2.5 μL start solution (200 mM $MgCl_2$, 200 μM ATP, 3 μCi [0.111 MBq] γ-^{32}P-ATP; Amersham Pharmacia). After incubation for 10 minutes, the reaction was terminated with the addition of 100 μL stop solution (chloroform, methanol, and 11.6 N HCl in the ratio of 50:100:1). Labeled phosphoinositides were extracted using chloroform, washed 3 times in 100 μL wash solution (mixture of 50 μL methanol and 50 μL 1 N HCl), separated by thin-layer chromatography, and analyzed using a BAS 2000 bioimaging analyzer (Fuji, Tokyo, Japan).

Results

The p85α protein is the major class IA PI3K isoform expressed in platelets

To explore the relative contributions of the various isoforms of class IA PI3K, we first examined the expression of various isoforms in murine platelets by Western blotting using anti-$p85^{PAN}$ antiserum, which has been shown to recognize all variants of p85α (p85α, p55α, and p50α), p85β, and p55γ.[23-25] Among the variety of hematopoietic tissues examined, expression levels of p85α isoform far exceeded those of p55α and p50α in platelets, spleen, thymus, and fetal liver, whereas bone marrow expressed similar amounts of all 3 isoforms (Figure 1A). In platelets from p85α−/− mice, selective disruption of the p85α isoform did not result in significant up-regulation of p55α, p50α, and p85β proteins to compensate for the lack of p85α protein (Figure 1B).[24,25] In addition, expression patterns of p85α, p55α, and p50α isoforms in p85α+/− platelets did not significantly differ from those of p85α+/+ platelets (Figure 1B). The p85β protein was detected in p85α−/− platelets only when the film was overexposed (data not shown). When expression of the p85β protein in platelets was examined using a specific antibody, the levels of p85β in p85α−/− and p85β+/+ platelets were found to be not significantly different (Figure 1C). It was also found that the $p85^{PAN}$ antiserum equally and efficiently captured p85β proteins in platelet lysates from either p85α+/+ or

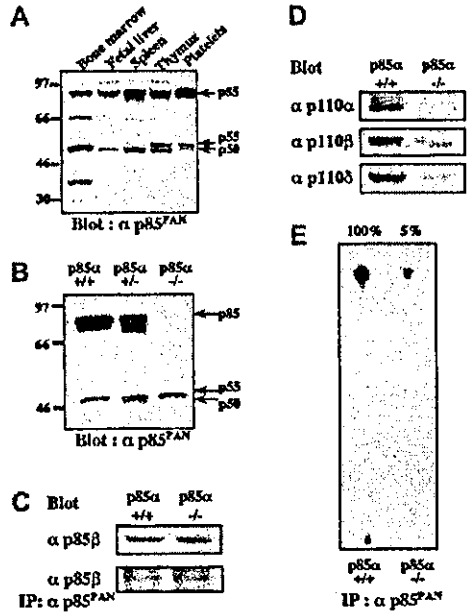

Figure 1. Differential expression patterns of regulatory and catalytic subunits of PI3K class IA and their kinase activities in platelets from p85α−/− and wild-type mice. (A-B) Expression patterns of regulatory subunits of PI3K class IA in wild-type hematopoietic organs (A) and in platelets from wild-type p85α+/+, p85α+/−, and p85α−/− mice (B), shown by Western blot with $\alpha p85^{PAN}$. (C-D) The amount of p85β protein in platelet lysate and its immunoprecipitate with anti-$p85^{PAN}$ antiserum (C) and the amount of p110 catalytic subunits (p110α, p110β, and p110δ) in platelet lysate from p85α+/+ and p85α−/− mice (D), shown by Western blot with p85β-specific antiserum and anti-p110 polyclonal antibodies, respectively. Whole-cell lysate (50 μg per lane) or its immunoprecipitate was resolved using SDS-PAGE. (E) PI3K activity of wild-type and p85α−/− platelets measured using anti-$p85^{PAN}$ antiserum and resolved by thin-layer chromatography. The wild-type response was set to 100% and the activity of p85α−/− platelets was estimated to be 5% of the wild-type response.

p85α−/− mice (Figure 1C). In addition, the expression of catalytic subunit p110α in p85α−/− compared with p85α+/+ platelets was almost undetectable, while expression of p110β and p110δ subunits were greatly reduced in amount, consistent with the instability of the p110 proteins in the absence of sufficient adaptor subunit concentrations (Figure 1D).[27] Consistent with these observations, remaining class IA PI3K activity in p85α−/− platelets was only 5% of the activity in wild-type littermates (Figure 1E).

PI3K p85α-deficient mice displayed perturbation of platelet aggregation response to collagen and CRP, but no bleeding disorders

In contrast to Syk-, SLP-76–, or PLCγ2-deficient mice, PI3K p85α−/− mice were born intact, with no bleeding disorders.[28-30] Bleeding times for PI3K p85α−/− mice (178 ± 89 seconds, n = 5) were not significantly prolonged compared with littermate controls (216 ± 97 seconds, n = 5), and peripheral blood cell counts were indistinguishable among wild-type, p85α+/−, and p85α−/− mice (data not shown). These results suggest that p85α deficiency does not cause profound defects in platelet production or function in vivo. These observations prompted us to examine the effect of PI3K p85α deficiency on platelet function in vitro.

Since many studies have suggested that the involvement of PI3K in signaling cascades in platelets is stimulated by various types of agonists, we investigated the platelet aggregation response in knockout mice. As shown in Figure 2, p85α deficiency led to an approximate 40% to 60% reduction of platelet aggregation in response to suboptimal or optimal concentrations of collagen (10 and 20 μg/mL, respectively) or the GP VI–specific agonist CRP (2.5 and 5 μg/mL). In particular, responses to lower dose stimuli, such as 5 μg/mL collagen or 1 μg/mL CRP, which induced 60% to 80% aggregation in wild-type platelets, were completely abrogated in p85α−/− mice. In contrast, responses to ADP, the thromboxane A_2 analog U46619, thrombin, PMA, A23187, or botrocetin were all intact in p85α−/− mice compared with littermate controls, even at subthreshold concentrations. The same responses to collagen and CRP were observed in both p85α+/− mice and wild-type littermates (data not shown). These results are consistent with previous in vitro observations in humans that class IA PI3K plays an important role in collagen-induced platelet signaling through GP VI engagement.[12,13]

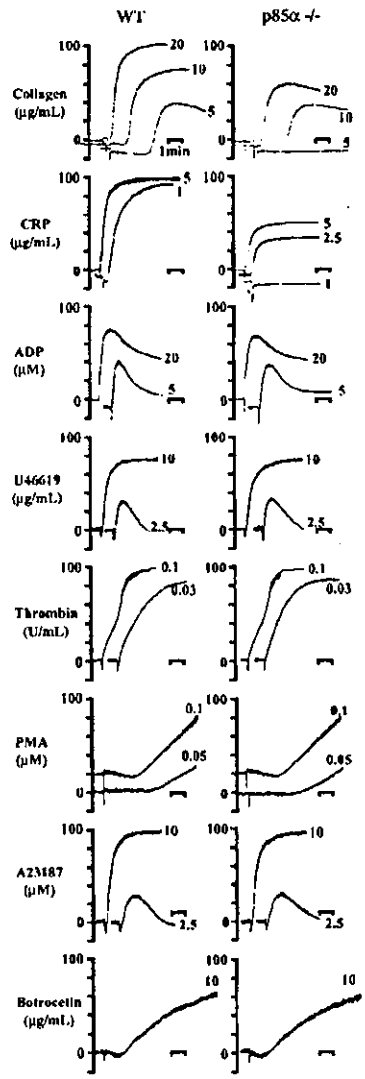

Figure 2. Platelet aggregation responses to various kinds of agonists. PRPs (3 × 10^5/μL) were incubated at 37°C for 10 minutes prior to stimulation. Changes to morphology and aggregation of platelets from wild-type (WT, left column) and p85α−/− (right column) were measured using an aggregometer after stimulation with collagen, CRP, ADP, U46619, thrombin, PMA, A23187, and botrocetin at the indicated concentrations. Bars indicate 1 minute. Results are from 1 experiment but are representative of at least 4 separate experiments.

PI3K p85α deficiency led to impaired P-selectin expression or fibrinogen binding in response to CRP

Next we analyzed the impact of p85α deficiency on platelet signaling events induced by GP VI activation. We first investigated whether p85α deficiency affects P-selectin expression or fibrinogen binding in response to CRP stimulation. As shown in Figure 3, panels A and B, 5 μg/mL CRP induced an approximately 10-fold increase in P-selectin expression in wild-type platelets, while expression in p85α−/− platelets was approximately 50% of the wild-type level. The impaired P-selectin expression in p85α−/− platelets was observed at CRP concentrations ranging from 0.01 to 5 μg/mL. Similarly, CRP-induced fibrinogen binding to p85α−/− platelets was significantly impaired (Figure 3C-D).

PI3K p85α-deficient platelets displayed impaired spreading over collagen- or CRP-coated surfaces

In order to further elucidate the role of p85α in collagen-induced platelet signaling, the adhesive response of platelets from knockout mice was investigated. Platelets were placed on collagen- or CRP-coated plates and allowed to adhere to the surface; then morphology was analyzed under scanning electron microscopy. As shown in Figure 4A, wild-type platelets adhered and spread over collagen- or CRP-coated surfaces after 90 minutes of incubation. In contrast, platelets from p85α−/− mice demonstrated reduced spreading, although filopodial protrusions were relatively intact (Figure 4A). Compared with the collagen-coated plates, poor lamellae formation in p85α−/− platelets was pronounced on the CRP-coated plates, consistent with the relative specificity of this isozyme to GP VI pathways among the multiple signaling cascades stimulated by collagen. Indeed, compared with wild-type platelets, filopodia on adhered platelets from p85α−/− mice had increased numbers and length (Figure 4B). These data suggest that p85α

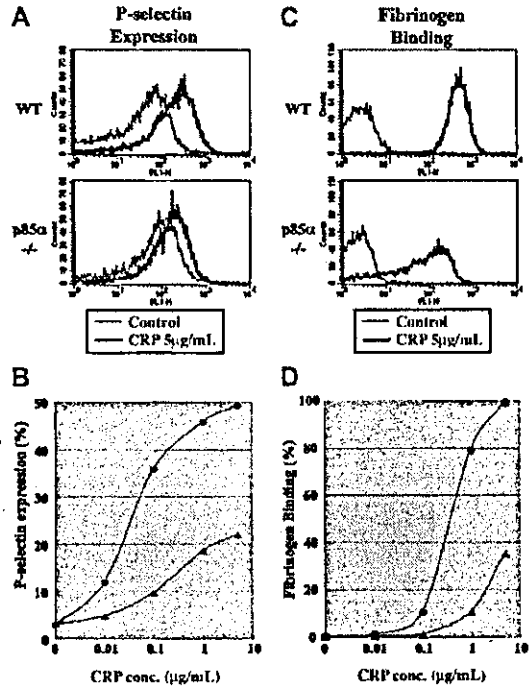

Figure 3. Surface expression of P-selectin on platelets and fibrinogen binding to platelets induced by GP VI stimulation. (A-B) P-selectin expression was detected using FITC-conjugated antimouse P-selectin antibody (A) and analyzed by flow cytometry (B). (C-D) Fibrinogen binding was detected using activated integrin $\alpha_{IIb}\beta_3$ and binding of Alexa Fluor 488–conjugated human fibrinogen (C) and analyzed by flow cytometry (D). Washed wild-type (WT) and p85α−/− platelets suspended in modified Tyrode-HEPES buffer containing apyrase and RGDS peptide with 1 mM $CaCl_2$ (A-B) or modified Tyrode-HEPES buffer containing apyrase with 1 mM $CaCl_2$ (C-D) were stimulated by CRP. Data are from 1 experiment but are representative of 3 independent experiments. In panels B and D, ● indicates WT platelets and ▲ indicates p85α−/− platelets.

plays an essential role in platelet lamellipodia formation during adhesive responses triggered by GP VI engagement.

Collagen and CRP induce a reduction in tyrosine phosphorylation of PLCγ2 and several other downstream molecules in PI3K p85α-deficient platelets

Collagen and CRP induced tyrosine phosphorylation of various signaling molecules, including Syk, LAT, SLP-76, Btk, Tec, Akt/protein kinase B (PKB), and PLCγ2 in platelets.[11,13,28-34] To investigate the underlying molecular mechanisms of impaired platelet function mediated by the GP VI/FcRγ complex in PI3K p85α–deficient mice, we analyzed tyrosine phosphorylation of these signaling molecules in response to collagen and CRP. Key molecules, Syk, LAT, and SLP-76, initially recruited and activated in the vicinity of the GP VI/FcRγ complex, were equally phosphorylated by CRP in both wild-type and p85α−/− platelets (Figure 5A-C). In contrast, phosphorylation of Btk, Tec, or Akt in downstream PH domain–containing effectors for PI3K in response to CRP was partially abrogated in p85α−/− mice (Figure 5D-F). Similar results were also obtained using collagen as the GP VI stimulator (data not shown). It is well known that PLCγ2 is one of the critical targets of collagen- and CRP-induced signaling in platelets. As shown in Figure 5G, phosphorylation of PLCγ2 was also clearly decreased in p85α−/− platelets.

These results suggest that the impaired response to collagen and CRP seen in p85α-deficient platelets is, at least partially, based on reduced phosphorylation and/or activation of PLCγ2.

Discussion

This report provides the first direct evidence of the effect of PI3K p85α deficiency on platelet function in vivo, demonstrating that class IA PI3K functions exclusively as a major element in the GP VI/FcRγ complex–mediated signaling cascade in mice. In contrast, the fact that this enzyme subclass did not exert any significant effect on other relevant platelet signaling pathways, such as those triggered by thrombin, ADP, U46619, and botrocetin, was rather unexpected.

GP VI ligation by collagen or CRP initiates intracellular signals through phosphorylated ITAMs on the FcRγ chain, leading to activation of serially connected downstream molecules in a phosphotyrosine-dependent manner.[13] At the end of this signaling cascade, the fully activated PLCγ2 is assembled at or in the vicinity of membrane rafts with signaling complexes such as tyrosine kinases Lyn/Fyn, Syk, Btk/Tec, and adaptors of LAT and SLP-76.[35-37] Evidence of the participation of class IA PI3K in this particular signaling cascade is that in the absence of PI3K p85α, platelets become defective in the following GP VI–induced signaling events: platelet aggregation, degranulation of α granules, integrin activation, lamellipodia formation, and tyrosine phosphorylation of putative effector molecules. Consistent with this conclusion, Btk mutations in humans[32,33] and Syk, LAT, SLP-76, and

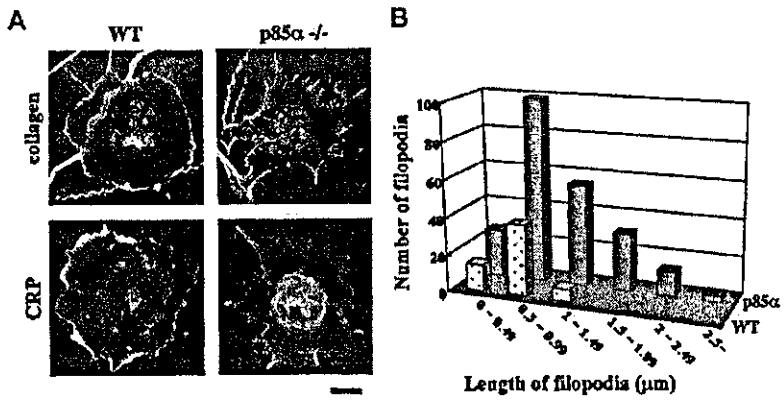

Figure 4. Morphologic examination of platelets adhering to collagen- or CRP-coated surfaces. (A) Washed platelets suspended in modified Tyrode-HEPES buffer containing apyrase and 2 mM $MgCl_2$ were exposed to surfaces coated with collagen (top row) or CRP (bottom row). Scanning electron images show wild-type (WT; left panels) and 85α−/− (right panels) platelets after 90 minutes of incubation. Scale bar indicates 1 μm. (B) Frequency analysis of the length of remnant filopodia from wild-type (dotted columns) and p85α−/− (solid columns) platelets (n = 50) adhering to CRP-coated surface after 60 minutes. Analysis was performed using NIH Image software.

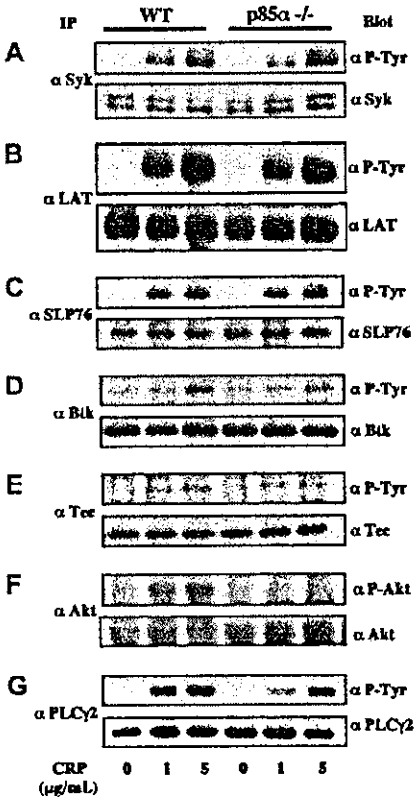

Figure 5. Protein phosphorylation in GP VI–stimulated wild-type and PI3K p85α−/− platelets. Murine platelets were treated using 0.2 mM acetylsalicylic acid and suspended in modified Tyrode-HEPES buffer containing 0.4 U/mL apyrase, 1 mM RGDS peptide, and 1 mM EGTA. The platelets were stimulated with CRP at 0, 1, and 5 μg/mL on an aggregometer with constant stirring and were lysed 90 seconds after stimulation. Then they were subjected to immunoprecipitation using anti-Syk (A), anti-LAT (B), anti–SLP-76 (C), anti-Btk (D), anti-Tec (E), anti-Akt (F), and anti-PLCγ2 (G) antibodies. Proteins were resolved using SDS-PAGE, transferred to nitrocellulose membrane, immunoblotted with antiphosphotyrosine (P-Tyr) antibody 4G10 or anti–phospho-Akt–specific antibody, and reprobed with the antibodies used for immunoprecipitation to demonstrate equal amounts of immunoprecipitated proteins in each lane. Identical results were obtained in at least 4 separate experiments.

PLCγ2 mutations in mice[28-31] all produce platelet phenotypes similar to those seen in p85α-deficient mice.

The finding that GP VI–induced tyrosine phosphorylation of Syk, LAT, and SLP-76 is not defective in p85α-deficient platelets might indicate that these molecules are active upstream or are independent of the PI3K-pathway. In fact, the p85 subunit of class IA PI3K has been shown to associate with tyrosine-phosphorylated LAT and tyrosine-phosphorylated ITAM of the FcRγ chain through each of the tandem SH2 domains following GP VI stimulation.[12] This association then triggers PI3K-pathway activation, resulting in the liberation of D3 phosphoinositides. LAT is tyrosine-phosphorylated by Syk following binding of the kinase to the phosphorylated ITAM of the FcRγ chain. LAT then forms a complex with SLP-76 via the adaptor protein Gads, becoming a scaffold for PLCγ2 recruitment.[13,31] The PI3K lipid products target the Btk PH domain and the PLCγ2 SH2 or PH domains to induce subcellular localization.[13,38] Therefore, 2 independent pathways exist—one mediated by membrane protein LAT and the other by PI3K membrane lipid products—for the full activation of PLCγ2 at specific membrane microdomains. Keeping this model in mind, the finding that the loss of the p85α protein does not exert a profound effect on platelet function is not particularly surprising. Although PLCγ2 activity in p85α-deficient platelets was not measured in this study, we identified partial but substantial reduction in GP VI–induced tyrosine phosphorylation of PLCγ2 in p85α-deficient platelets, which may be due to the incomplete defect in platelet cellular responses following GP VI stimulation. Other groups have reported that treatment with pharmacologic PI3K inhibitors strongly suppresses GP VI–induced PLCγ2 activation but has a minimal effect on tyrosine phosphorylation in human platelets.[11,17] This discrepancy might be attributed to species differences or the methods of inducing PI3K disruption. The lipid kinase activity of class IA PI3K is almost completely deleted in p85α-deficient platelets. Only trace amounts of activity may be attributed to the very low levels of the other kinase isoforms associated with the p85α splice variants p55 and p50, p85β, or p55γ. Nevertheless, complete loss of p85α activity does not result in the severe platelet phenotype seen in response to GP VI stimulation, supporting the hypothesis that full activation of PLCγ2 may involve a PI3K-independent pathway, possibly via the scaffold complex formed by LAT, Gads, and SLP-76.[13,39] However, this concept is challenged by the finding that the extent and spectrum of immune deficiency resulting from the loss of p85α in mice B lymphocytes closely approximates those seen in PLCγ2-deficient mice.[25,30,40] In addition, mice with the Btk deficiency Xid (a naturally occurring point mutation in the PH domain of the kinase) are unable to bind PI3K products PtdIns-3,4,5-P3 or SLP-65/BLNK (an adaptor connecting Btk and PLCγ2 and thought to be a counterpart of the LAT/SLP-76 adaptor complex in T lymphocytes) and also display remarkably similar immune phenotypes. This indicates that p85α and PLCγ2 are serially connected through Btk and SLP-65/BLNK.[41-43] Although this discrepancy may simply be due to differences in cell type, careful comparisons of the platelet phenotypes of p85α-deficient mice and PLCγ2-deficient mice of identical genetic backgrounds are required to address the issue.

Partial reduction of GP VI–induced tyrosine phosphorylation of Btk and Tec in p85α-deficient platelets supports the previously proposed hypothesis that 2 PH domains containing tyrosine kinases are located proximally to class IA PI3K and function as redundant PI3K effectors in human platelets.[33] B lymphocytes from Tec-deficient mice exhibit no apparent immune phenotype, but the double deficiency of Btk and Tec results in a more severe disorder than the Btk deficiency alone.[44] Whether this is also true for murine platelets is yet to be determined. Since the immune phenotype of Xid mice (with normal levels of protein expression) is comparable to that of Btk-deficient animals, Btk activation should be virtually PI3K-dependent.[45] Despite nearly total loss of PI3K activity and expression in p85α-deficient platelets, minimal tyrosine phosphorylation of both kinases remains inducible by GP VI stimulation. This implies the existence of an alternate pathway for Btk/Tec activation that is independent of PI3K.[46,47]

The defective spreading of p85α-deficient platelets over collagen- or CRP-coated surfaces is most likely caused by the loss of PI3K products. Lamellipodia formation in adherent cells is mediated by Rac-1, a member of the Rho family of small guanosine triphosphatases (GTPases). This protein is regulated by a PH domain–containing GEF Vav family.[48,49] In fact, Vav-1, a member of this family, is present in high quantities in platelets.[48] In contrast, filopodial protrusion is mediated through the RhoA small GTPase with the help of cdc42 GEF, which is regulated in a PI3K-independent manner.[49] The observation that p85α-deficient platelets show defective lamellipodia formation while retaining intact filopodial protrusions is therefore unsurprising. In agreement with

these observations, treatment of human platelets with wortmannin and LY294002 followed by contact with a collagen- or CRP-coated surface reportedly results in similar phenotypic changes to platelet morphology.[17]

In p85α-deficient mice, the apparent lack of alteration in platelet aggregation response to other important platelet stimulators (thrombin, ADP, U46619, PMA, A23187, and botrocetin) was unexpected, since class IA PI3K involvement has been proposed to activate $\alpha_{IIb}\beta_3$ integrin (inside-out signaling) or assist in the postaggregation response through integrin engagement (outside-in signaling)[4,50] in the final common pathways. For example, thrombin- or PMA-induced accumulation of PtdIns-3,4-P2 or PtdIns-3,4,5-P3 and the concomitant up-regulation of $\alpha_{IIb}\beta_3$ integrin receptor function in human platelets are effectively inhibited by low nanomolar ranges of wortmannin, indicating that class IA PI3K is downstream of protein kinase C in the induction of integrin activation.[4,51,52] Following thrombin stimulation, the p85α subunit is reportedly translocated into the focal contact area of human platelets by association with the SH3 domain and the proline-rich region of the focal adhesion kinase p125FAK.[18] However, these observations disagree with our finding that p85α-deficient platelets display intact aggregation responses to thrombin and PMA, even at suboptimal concentrations. One possible explanation for the discrepancy may be the existence of a class II PI3K, which is also sensitive to wortmannin, downstream of $\alpha_{IIb}\beta_3$ integrin.[53] Activation of GPCRs triggered by weak agonists such as ADP and U46619 readily induces PI3K activity in platelets.[4,19,20] G protein βγ–specific PI3Kγ is the most plausible candidate for this particular pathway, and class IA PI3K is also postulated to be activated directly by G protein βγ subunits or tyrosine-phosphorylated intermediates following GPCR engagement.[9,21] Indeed, a recent study has shown that PI3K 110γ–deficient platelets exhibit impaired response to ADP, and the defect is limited to the Gi-coupled ADP receptor (P2Y12).[54] This finding, together with the observations of p85α-deficient platelets described herein, suggests that other PI3K species with involvement in the signaling pathway elicited by thrombin and U46619 or with redundant functions may exist.

The snake venom–derived botrocetin induces binding of von Willebrand factor via the receptor GP Ib/IX/V complex and mediates platelet-platelet interactions that may be accompanied by activation of class IA PI3K through either the cytoplasmic tail of the complex or ITAM-containing FcR (FcRγ in humans and mice and FcγRIIA in humans) colocalized with the complex.[16,55,56] However, we were unable to observe any perturbed aggregatory response to botrocetin, even at suboptimal concentrations. The role of p85α in the GP Ib/IX/V pathway thus requires further study.

In conclusion, absence of PI3K p85α in mice leads to compromised platelet responses to GP VI stimulation in vitro, but no significant bleeding disorder. Although class IA PI3K is reportedly involved in multiple signaling pathways or different stages of cellular process in platelets, the p85α isoform functions exclusively as a major component of the ITAM-mediated signaling pathway. Platelets lack a nucleus but retain a similar set of intracellular machinery for immune receptor signaling. The cells therefore represent appropriate targets for research to elucidate the mechanisms of immediate immune responses.

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PI3K and Btk differentially regulate B cell antigen receptor-mediated signal transduction

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Phosphoinositide-3 kinase (PI3K) is thought to activate the tyrosine kinase Btk. However, through analysis of PI3K$^{-/-}$ and Btk$^{-/-}$ mice, B cell antigen receptor (BCR)-induced activation of Btk in mouse B cells was found to be unaffected by PI3K inhibitors or by a lack of PI3K. Consistent with this observation, PI3K$^{-/-}$ Btk$^{-/-}$ double-deficient mice had more severe defects than either single-mutant mouse. NF-κB activation along with Bcl-x$_L$ and cyclin D2 induction were severely blocked in both PI3K$^{-/-}$ and Btk$^{-/-}$ single-deficient B cells. Transgenic expression of Bcl-x$_L$ restored the development and BCR-induced proliferation of B cells in PI3K$^{-/-}$ mice. Our results indicate that PI3K and Btk have unique roles in proximal BCR signaling and that they have a common target further downstream in the activation of NF-κB.

Phosphoinositide-3 kinase (PI3K) is a key enzyme producing phospholipid second messengers and has an important role in various signal transduction pathways[1,2]. PI3K family members are classified into three groups according to their structure and substrate specificity[2]. Among them, class I$_A$ heterodimeric PI3Ks consisting of a catalytic subunit (p110α, p110β, p110δ) and a regulatory subunit (p85α, p85β, p55γ) are involved in receptor-mediated signaling in the immune system. To precisely examine the functions of class I$_A$ PI3Ks, we and others generated PI3K$^{-/-}$ mice deficient for the gene encoding p85α, the most abundantly and ubiquitously expressed regulatory subunit of class I$_A$ PI3Ks[3-5]. Due to alternative splicing, p55α and p50α, in addition to p85α, are produced from the same gene[6,7]. Mice lacking only p85α (used here as PI3K$^{-/-}$ mice) are viable[3,4], whereas mice lacking all alternatively spliced products are unable to survive after birth[5]. In the absence of PI3K, B cell development from pro-B cells to pre-B cells in the bone marrow is impaired and the number of mature B cells in the periphery is decreased[4,5]. In addition, mature B cell functions such as mitogen-induced proliferation *in vitro* are severely impaired[4].

Crosslinking of the surface B cell antigen receptor (BCR) evokes sequential activation of a variety of protein and lipid kinases including Src family kinases (Lyn, Fyn, Blk), Syk, Btk, Akt (also known as PKB) and PI3K[2,8-12]. Although activation of PI3K is observed upon BCR stimulation, signaling events upstream and downstream of PI3K are not well characterized. In B cells, Lyn, c-Cbl, CD19 and BCAP bind the p85α subunit of PI3K, suggesting that these molecules are upstream activators of PI3K. On the other hand, various proteins containing pleckstrin homology (PH) domains, such as Akt, phosphoinositide-dependent kinase 1 (PDK1) and Btk, are thought to function downstream of PI3K, because of the ability of their PH domains to bind phosphatidylinositol-(3,4)-bisphosphate (PIP$_2$) or phosphatidylinositol-(3,4,5)-trisphosphate (PIP$_3$), products of PI3K[13,14].

Btk, a Tec family kinase, is activated by tyrosine phosphorylation and has a critical role in BCR signaling[12,15-18]. Btk$^{-/-}$ mice, as well as mice with the *Xid* mutation (a natural mutation in the PH domain of Btk in which an arginine residue critical for the binding to PIP$_3$ is replaced by cysteine), show deficiencies in the development and activation of B cells. In humans, deficiency of Btk leads to X-linked Bruton's type agammaglobulinemia (XLA)[12,15,16]. Stimulation-dependent membrane localization of a Btk-PH domain-GFP chimeric protein in transient transfection systems has been demonstrated and such membrane recruitment is blocked by wortmannin, a PI3K inhibitor[19,20]. Overexpression of the p110 PI3K catalytic subunit in a B cell line results in Btk tyrosine phosphorylation[21]. It has been proposed from these observations that PI3K is responsible for the activation of Btk by bringing Btk to the plasma membrane through interactions between the PH domain of Btk and PIP$_3$[13,14], leading to tyrosine phosphorylation of Btk by other protein tyrosine kinases such as Syk. It was thus not surprising that PI3K$^{-/-}$ mice show a phenotype similar to that of Btk$^{-/-}$ or *Xid* mice[4,5].

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BCR stimulation also activates the serine-threonine kinase Akt[2,22]. Akt has crucial roles in anti-apoptotic signal transduction as well as cell cycle progression[23-25]. Activation of Akt prevents apoptosis in many cell types and its anti-apoptotic effect is blocked by wortmannin. One product of PI3K, PIP_2, is reported to associate with the PH domain of Akt to recruit the enzyme to the plasma membrane. Similarly, another PI3K product, PIP_3, recruits PDK1, which phosphorylates Akt to activate its kinase activity. Akt is the major downstream target of PI3K in many signal transduction pathways[2,23-26].

Here, we further investigated the role of PI3K in B cell signal transduction pathways and the functional relationship between PI3K and Btk, using $PI3K^{-/-}$ and $Btk^{-/-}$ mice. Contrary to our expectations, BCR-induced activation of Btk was unaffected by the lack of PI3K or by PI3K inhibitors. On the other hand, BCR-induced activation of Akt was normal in $Btk^{-/-}$ B cells, but was severely impaired in $PI3K^{-/-}$ B cells. Furthermore, $PI3K^{-/-}Btk^{-/-}$ double-deficient mice show more severe phenotypes than either single-deficient mouse. These biochemical and genetic data show that PI3K and Btk function independently in BCR signal transduction pathways. Among downstream events, activation of NF-κB and induction of Bcl-x_L and cyclin D2 were impaired in both $PI3K^{-/-}$ and $Btk^{-/-}$ single-deficient B cells. Forced expression of Bcl-x_L restored development and proliferative responses of B cells in $PI3K^{-/-}$ mice. Our results indicate that class I_A PI3K and Btk have clearly distinct roles in BCR signal transduction.

Results

PI3K-dependent activation of Akt upon BCR stimulation

B cells from $PI3K^{-/-}$ mice used in this study expressed low amounts of p50α[4]. Expression of p85β and p55γ regulatory subunits was very low or undetectable in $PI3K^{-/-}$ and wild-type (WT) B cells (**Fig. 1a**). Expression of p110δ, the most abundantly expressed catalytic subunit in B cells, was reduced in the absence of these regulatory subunits (**Fig. 1a**). BCR-dependent activation of PI3K in the absence of p85α was examined by *in vitro* kinase assay, using phosphatidylinositol as a substrate to detect generation of phosphatidylinositol-3-phosphate (**Fig. 1b**). Total PI3K activity in tyrosine phosphorylated proteins was increased by BCR stimulation in WT and $Btk^{-/-}$ mice. In contrast, only a small amount of PI3K activity was observed upon BCR stimulation in $PI3K^{-/-}$ B cells (**Fig. 1b**; ~5% of WT activity), as previously reported[4]. The p50α regulatory subunit and possibly another class of PI3K likely contribute to this residual increase of PI3K activity in $PI3K^{-/-}$ B cells. On the contrary, activation of PI3K was unaffected in $Btk^{-/-}$ B cells.

Because Akt is widely accepted as a downstream target of PI3K in B cell signal transduction[2,22-26], we investigated BCR-mediated Akt activation by immunoblotting with specific monoclonal antibodies (mAbs) that detect phosphorylation at residues Thr^{308} and Ser^{473} of Akt, which is known to correlate with its kinase activity[27,28]. Phosphorylation of Akt on the Thr^{308} and Ser^{473} residues was increased upon BCR stimulation after 5 min in WT B cells, whereas phosphorylation of Akt

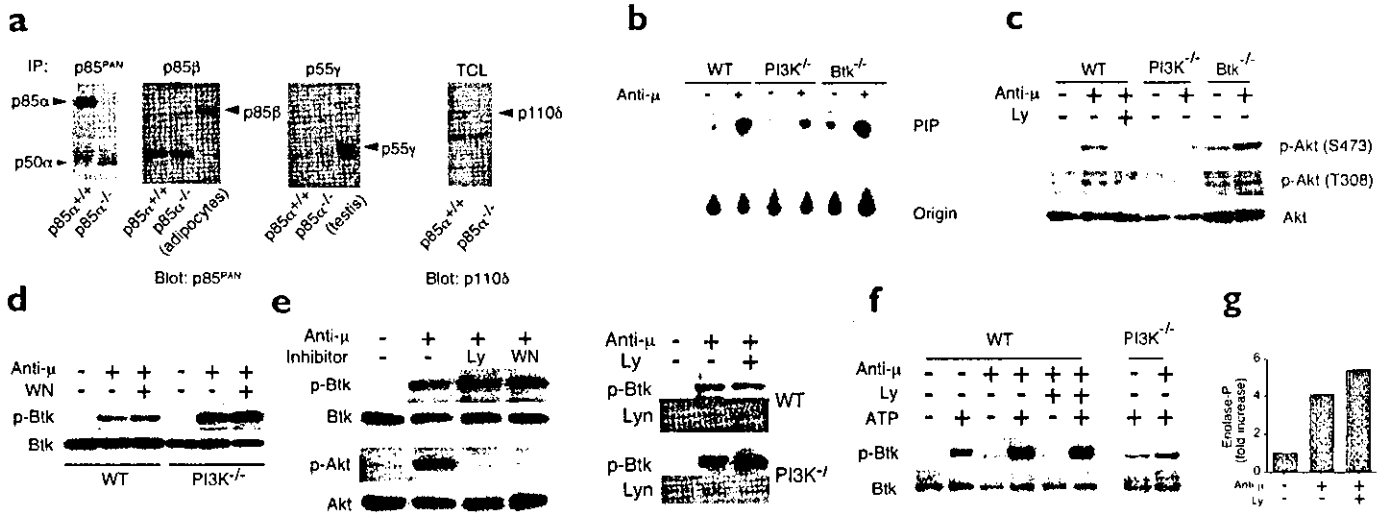

Figure 1. The Akt, but not the Btk, pathway is dependent on PI3K in B cells. **(a)** Expression of PI3K regulatory subunits in $PI3K^{-/-}$ ($p85\alpha^{-/-}$) and WT ($p85\alpha^{+/+}$) B cells. Postnuclear lysates of B cells derived from the indicated mice were immunoprecipitated with anti-$p85^{PAN}$ and specific antisera for p85β and p55γ, then immunoblotted with anti-$p85^{PAN}$. Adipocytes and testis were used as positive controls for p85β and p55γ, respectively. Or, total cell lysates (TCL) prepared from $PI3K^{-/-}$ ($p85\alpha^{-/-}$) and WT ($p85\alpha^{+/+}$) B cells were immunoblotted with anti-p110δ. **(b)** PI3K activities in $PI3K^{-/-}$, $Btk^{-/-}$ and WT B cells. PI3K activities from BCR-stimulated B cells of the indicated genotypes were assayed. **(c)** PI3K-dependent activation of Akt. BCR-mediated activation of Akt in $PI3K^{-/-}$ and $Btk^{-/-}$ B cells was evaluated by immunoblotting with a specific antibody detecting phosphorylation at Thr^{308} (p-Akt (T308)) and Ser^{473} (p-Akt (S473)) residues of Akt. Membranes were re-blotted with anti-Akt (Akt). Data are representative of four independent experiments with similar results. **(d)** BCR-induced tyrosine phosphorylation of Btk in $PI3K^{-/-}$ B cells. WT and $PI3K^{-/-}$ B cells on a BALB/c background were stimulated with anti-IgM $F(ab)'_2$ (Anti-μ) in the presence or absence of 25 nM wortmannin (WN). Btk was then immunoprecipitated by anti-Btk and immunoblotted with 4G10 (p-Btk). Membranes were re-blotted with anti-Btk, 43-3B (Btk). **(e)** (Left) Effects of PI3K inhibitors on tyrosine phosphorylation of Btk. WT B cells were stimulated by BCR crosslinking (Anti-μ) in the absence or presence of 50 nM wortmannin (WN) or 25 μM Ly294002 (Ly). Btk was then immunoprecipitated and immunoblotted with 4G10 (p-Btk). Membranes were re-blotted with 43-3B (Btk). At the same time, cell lysates were examined for Akt phosphorylation by anti-phospho-Akt(S473) (p-Akt). Membranes were re-blotted with anti-Akt (Akt). (Right) Membrane fractions were prepared from WT and $PI3K^{-/-}$ B cells unstimulated or stimulated by BCR crosslinking (Anti-μ) in the absence or presence of 25 μM Ly294002 (Ly), and examined for tyrosine phoshorylation (p-Btk). Membranes were re-blotted with anti-Lyn (Lyn). Data in **(d)** and **(e)** are representative of three independent experiments with similar results. **(f,g)** BCR-induced activation of Btk. WT and $PI3K^{-/-}$ B cells on a BALB/c background were stimulated with or without 20 μg/ml of anti-IgM $F(ab)'_2$ (Anti-μ) at 37 °C for 3 min in the presence or absence of 10 μM Ly294002 (Ly). **(f)** Btk was immunoprecipitated and incubated with or without 100 μM ATP at 22 °C for 5 min followed by immunoblot analysis with 4G10. **(g)** Immunoprecipitates were incubated with acid-denatured enolase as an exogenous substrate in the presence of 100 μM ATP at 22 °C for 5 min. Btk activities are presented as the fold increase in the level of tyrosine phosphorylation of enolase. Data in **(f)** and **(g)** are representative of two independent experiments with similar results.

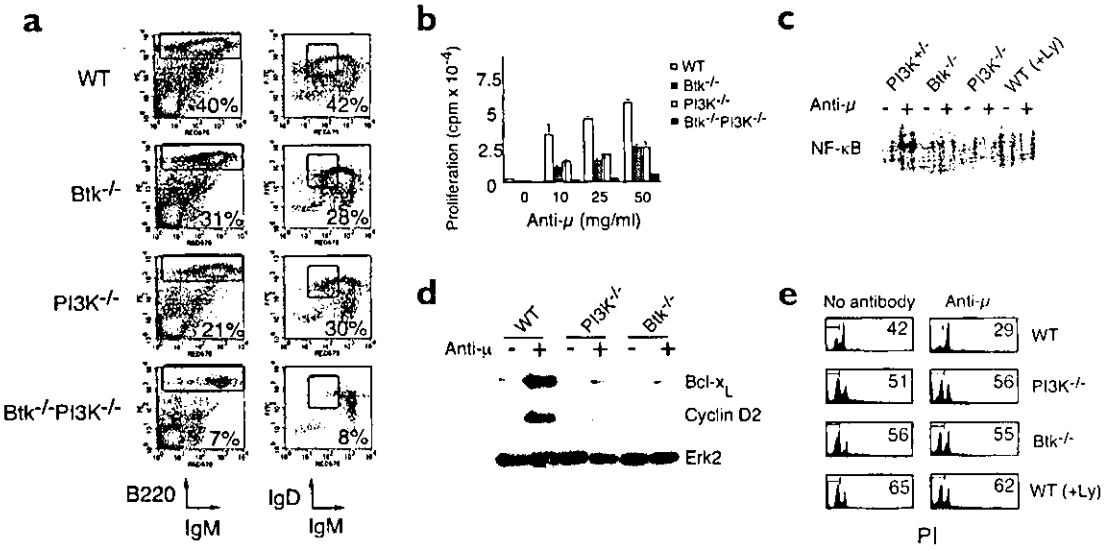

Figure 2. Phenotypes of PI3K and Btk double-deficient mice. (**a**) Splenocytes of indicated mice were stained with FITC-conjugated anti-IgD, PE-conjugated anti-B220 and biotinylated anti-IgM followed by Red670-conjugated streptavidin and examined by flow cytometry. IgM *versus* B220 profiles are shown on the left and IgM *versus* IgD profiles among B220$^+$ cells are shown on the right. Boxes in the left and right panels indicate total B cell and IgM^{lo}-IgD^{hi} circulating B cell fractions, respectively. (**b**) Proliferative responses of splenic B cells upon BCR stimulation *in vitro*. Proliferative responses are shown as [^{3}H]thymidine incorporation. Data are representative of two independent experiments with similar results. (**c**) NF-κB activation. PI3K$^{-/-}$ and Btk$^{-/-}$ B cells were stimulated with anti-μ in the absence or presence of Ly294002 (+Ly) for 4 h and nuclear extracts prepared. EMSA was carried out using ^{32}P-labeled NF-κB probe. (**d**) Induction of Bcl-x$_L$ and cyclin D2. Purified B cells of the indicated mice were stimulated with anti-μ for 16 h and evaluated for the expression of Bcl-x$_L$ and cyclin D2 by immunoblotting using specific antibodies. Membrane was re-blotted with anti-Erk2 (Erk2). (**e**) Apoptotic cell death in suspension culture. WT, PI3K$^{+/-}$ and Btk$^{-/-}$ B cells in the absence or presence of Ly294002 (+Ly) were incubated for 18 h and cell death was evaluated by DNA content analysis using propidium iodide. Numbers indicate the proportion of cells in the sub-G1 fraction (%) in cell cycle analysis. Data are representative of three independent experiments with similar results.

was severely blocked in the absence of PI3K (**Fig. 1c**). In contrast, BCR-mediated phosphorylation of Akt was unaffected in Btk$^{-/-}$ B cells (**Fig. 1c**), as shown previously[29]. Thus, BCR-mediated activation of Akt depends on PI3K, but not on Btk.

Activation of Btk is independent of PI3K

The phenotypic resemblance between PI3K$^{-/-}$ and Btk$^{-/-}$ mice in B cell developmental and activation defects suggests functional association between PI3K and Btk in BCR-mediated signal transduction[4,5]. If PI3K functions directly, and only, upstream of Btk, activation of Btk upon BCR stimulation would be expected to be impaired in PI3K$^{-/-}$ B cells. To this end, we examined the activation of Btk upon BCR stimulation (**Fig. 1d,e**).

First, purified B cells from PI3K$^{-/-}$ and WT mice were stimulated with a F(ab)'$_2$ fragment of anti-IgM and activation of immunoprecipitated Btk was evaluated by immunoblotting with the phosphotyrosine-specific mAb, 4G10. Contrary to our expectation, tyrosine phosphorylation of Btk induced by BCR crosslinking was unaffected in the absence of PI3K (**Fig. 1d**). Furthermore, addition of wortmannin had little effect on tyrosine phosphorylation of Btk in both PI3K$^{-/-}$ and WT B cells. Another PI3K inhibitor, Ly294002 also showed no effect on tyrosine phosphorylation of Btk (**Fig. 1e**, left). Both 50 nM wortmannin and 25 μM Ly294002, which inhibit all types of PI3Ks, did not block tyrosine phosphorylation of Btk, whereas these inhibitors completely block Akt activation in the same cells (**Fig. 1e**, left). Recruitment of phosphorylated Btk to the plasma membrane was also unaffected by inhibition of PI3K or by the lack of PI3K (**Fig. 1e**, right).

Next, we directly examined the kinase activity of Btk using an *in vitro* kinase assay system. BCR-induced activation of Btk activity, as examined by autophosphorylation of Btk, was unaffected in PI3K$^{-/-}$ B cells or by PI3K inhibitors (**Fig. 1f**). Likewise, Btk activation, as examined by phosphorylation of an exogenous substrate, enolase, was observed in the presence of Ly294002 (**Fig. 1g**). These results indicate that Btk can be activated in the absence of PI3K activity.

Phenotypes of PI3K$^{-/-}$Btk$^{-/-}$ double-deficient mice

To further examine if the activation of Btk can occur independent of PI3K in BCR signal transduction pathways, we used a genetic approach by comparing the phenotypes of single-deficient mice and PI3K$^{-/-}$Btk$^{-/-}$ double-deficient mice. If PI3K simply functions upstream of Btk by providing PIP$_3$ to the PH domain of Btk, the phenotype of double-deficient mice would be identical to that of PI3K or Btk single-deficient mice. On the other hand, if PI3K and Btk function independently in BCR signal transduction pathways, double-deficient mice should show a more severe phenotype. To this end, PI3K$^{-/-}$ and Btk$^{-/-}$ mice were crossed and analyzed. The number of mature (B220$^+$, IgM$^+$) splenic B cells in PI3K$^{-/-}$Btk$^{-/-}$ double-mutant mice was significantly ($P < 0.05$) less than that of each single-mutant counterpart (**Fig. 2a** and **Table 1**).

Table 1. Lymphocyte numbers in the spleen of Btk$^{-/-}$, PI3K$^{-/-}$ and PI3K$^{-/-}$Btk$^{-/-}$ mice

Genotype[a]	No. of B cells (× 10^6)	No. of IgM B cells (× 10^6)	No. of T cells (× 10^6)	B/T cell ratio
WT (n = 5)	28.3 ± 1.8	10.8 ± 1.0	28.7 ± 1.8	1.0 ± 0.1
Btk$^{-/-}$ (n = 7)	10.0 ± 2.4[b]	1.6 ± 1.5[e]	16.4 ± 2.0[h]	0.62 ± 0.17[j]
PI3K$^{-/-}$ (n = 5)	11.7 ± 4.8[b]	5.0 ± 3.4[e,f]	19.3 ± 5.8[h]	0.64 ± 0.28[j]
PI3K$^{-/-}$Btk$^{-/-}$ (n = 3)	6.2 ± 1.4[b,c,d]	1.0 ± 0.6[e,g]	22.5 ± 4.7	0.28 ± 0.06[i,k]

[a]Mice are on a mixed background between C57BL/6 and 129/Sv. Significance examined by Student-Newman-Keuls test: [b]$P < 0.01$ from WT; [c]$P < 0.05$ from Btk$^{-/-}$; [d]$P < 0.05$ from PI3K$^{-/-}$; [e]$P < 0.01$ from WT; [f]$P < 0.05$ from Btk$^{-/-}$; [g]$P < 0.05$ from PI3K$^{-/-}$; [h]$P < 0.01$ from WT; [i]$P < 0.05$ from WT; [j]$P < 0.01$ from WT; [k]$P < 0.05$ from Btk$^{-/-}$; [l]$P < 0.05$ from PI3K$^{-/-}$. Essentially the same results were obtained by statistical analysis using the Bonferroni correction method.

The number of circulating (B220$^+$, IgM^{low}, IgD^{high}) B cells among mature (B220$^+$, IgM$^+$) B cells in the spleen of PI3K$^{-/-}$Btk$^{-/-}$ double-deficient mice was also significantly ($P < 0.05$) lower than that in PI3K$^{-/-}$ mice, but was similar to that of Btk$^{-/-}$ mice (**Table 1**). When B/T cell ratios were compared, double-deficient mice show significantly ($P < 0.05$) lower B/T ratios than do single-deficient mice.

We next investigated the proliferative response of double-deficient B cells. Although BCR-induced proliferation of splenic B cells was impaired in PI3K$^{-/-}$ or Btk$^{-/-}$ mice, the response of double-deficient B cells was even lower than that of single-mutant B cells (**Fig. 2b**). These genetic data support the biochemical evidence that PI3K and Btk function independently in B cell signal transduction pathways.

Impaired induction of NF-κB and Bcl-x$_L$

BCR stimulation activates the NF-κB pathway and both Akt and Btk are involved in NF-κB activation in B cells[30–33]. We thus investigated BCR-mediated activation of NF-κB in PI3K$^{-/-}$ and Btk$^{-/-}$ B cells. In normal B cells, the activity of nuclear NF-κB complexes containing p50 and c-Rel was increased upon BCR stimulation as revealed by electrophoretic mobility shift assay (EMSA) analysis (**Fig. 2c** and data not shown). On the contrary, activation of NF-κB was reduced in PI3K$^{-/-}$ B cells and in Ly294002-treated WT B cells, indicating that BCR-dependent NF-κB activation involves the PI3K pathway. BCR-mediated activation of NF-κB was also blocked in Btk$^{-/-}$ B cells (**Fig. 2c**), as previously reported[31,32]. Thus, BCR-dependent activation of NF-κB requires both PI3K and Btk.

NF-κB is known to have a role in the induction of Bcl-x$_L$ and cyclin D2 upon BCR stimulation[34,35]. Bcl-x$_L$ induction after BCR stimulation was impaired in PI3K$^{-/-}$ and Btk$^{-/-}$ B cells (**Fig. 2d**). Furthermore, induction of cyclin D2, indicative of cell cycle progression, was blocked in both PI3K$^{-/-}$ and Btk$^{-/-}$ B cells (**Fig. 2d**), consistent with the observed BCR-induced proliferative responses (**Fig. 2b**). These results suggest that NF-κB-Bcl-x$_L$ and NF-κB-cyclin D2 pathways are common downstream targets of PI3K and Btk in BCR-mediated signal transduction.

As BCR-dependent induction of Bcl-x$_L$ was impaired in both PI3K$^{-/-}$ and Btk$^{-/-}$ mice, one prediction was that these mutant B cells would be more susceptible to apoptosis than WT B cells. We thus examined apoptotic cell death in suspension culture of PI3K$^{-/-}$ and Btk$^{-/-}$ B cells with or without BCR stimulation. Apoptotic death after an 18-h incubation was evaluated by the proportion of cells in the sub-G1 fraction in cell cycle analysis using propidium iodide staining (**Fig. 2e**). We found that 40% of splenic B cells showed apoptosis after 18 h cultivation *in vitro* without stimulation, and such spontaneous cell death in suspension culture was enhanced in the absence of PI3K or Btk (**Fig. 2e**). Although BCR stimulation with anti-IgM F(ab)'$_2$ fragment results in a partial rescue of WT B cells from apoptosis, BCR stimulation was unable to rescue PI3K$^{-/-}$ and Btk$^{-/-}$ B cells (**Fig. 2e**). These results indicate both PI3K$^{-/-}$ and Btk$^{-/-}$ B cells have an increased sensitivity to cell death, possibly because of the failure of BCR-mediated Bcl-x$_L$ induction.

Forced expression of Bcl-x$_L$ in PI3K$^{-/-}$ B cells

As shown above, the inability to induce Bcl-x$_L$ may lead to the low B cell numbers as well as the low proliferative response of PI3K$^{-/-}$ and Btk$^{-/-}$ B cells, and may explain the phenotypic resemblance between PI3K$^{-/-}$ and Btk$^{-/-}$ mice. It has been shown that forced expression of Bcl-x$_L$ in *Xid* mice restores B cell development and proliferative responses[36]. We thus examined the effect of overexpression of Bcl-x$_L$ in PI3K$^{-/-}$ B cells by generating Bcl-x$_L$ transgenic PI3K$^{-/-}$ mice. Equivalent numbers of mature B cells and circulating B cells were found in Bcl-x$_L$ transgenic PI3K$^{-/-}$ mice and WT mice (**Fig. 3a** and **Table 2**). When B/T cell ratios were compared, it was also apparent that the transgenic expression of Bcl-x$_L$ in PI3K$^{-/-}$ mice restored the relative lymphocyte composition to that found in WT mice.

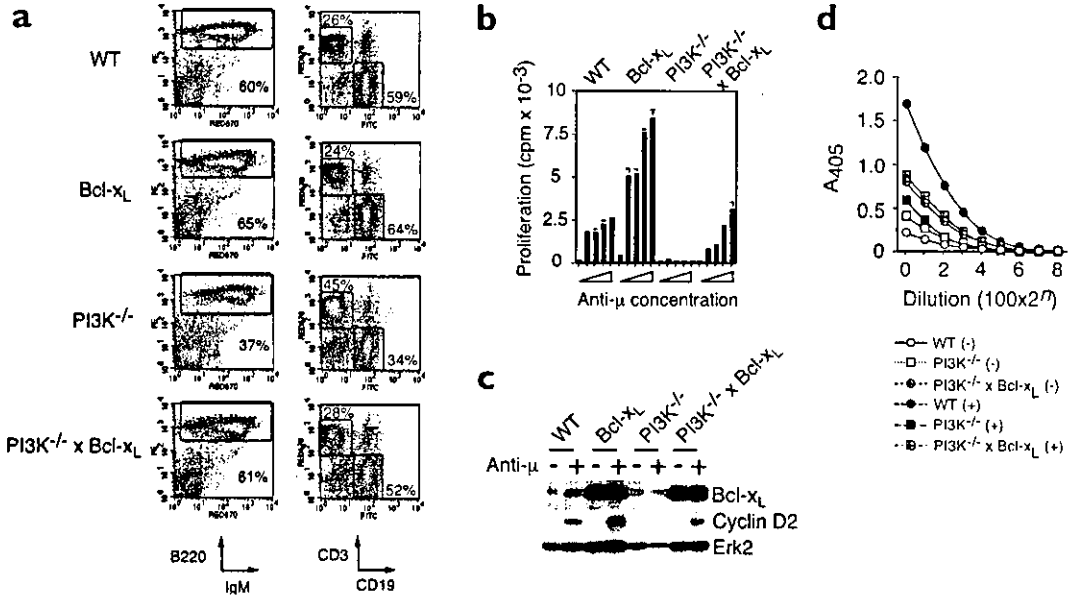

Figure 3. Restoration of B cell numbers and proliferative response of PI3K$^{-/-}$ mice by transgenic expression of Bcl-x$_L$. (**a**) Splenocytes of indicated mice were stained with a combination of PE-conjugated anti-B220 and biotinylated anti-IgM followed by Red670-conjugated streptavidin, or FITC-conjugated anti-CD19 and biotinylated anti-CD3 followed by Red670-conjugated streptavidin, and examined by flow cytometry. IgM *versus* B220 profiles are shown on the left and CD19 *versus* CD3 profiles to examine the ratio of B and T cells are shown on the right. Boxes in the left panels indicate B cell fractions. Top and bottom boxes in the right panels indicate T and B cell fractions, respectively. (**b**) Purified B cells of the indicated mice were examined for their proliferative responses following BCR stimulation as in **Fig. 2b**. Concentrations of anti-μ were 0, 5, 10, 25 and 50 μg/ml from left to right for each group. (**c**) Induction of cyclin D2 by forced expression of Bcl-x$_L$. Purified B cells of the indicated mice were stimulated with anti-BCR for 18 h and examined for the expression of Bcl-x$_L$ and cyclin D2. Note the constitutive expression of Bcl-x$_L$ in Bcl-x$_L$ transgenic B cells. Membrane was re-blotted with anti-Erk2 (Erk2). (**d**) T lymphocyte-independent antibody production of indicated mice using DNP-Ficoll was examined as described[3]. The immune sera (+) were analyzed at day 7 for DNP specific total immunoglobulin by ELISA and titers were shown as absorbance at 405-nm wavelength (A_{405}). Preimmune sera (-) were used as controls. Data are representative of two independent experiments with similar results.

Finally, we examined the functions of B cells in Bcl-x_L transgenic PI3K$^{-/-}$ mice. PI3K$^{-/-}$ B cells were incapable of proliferating in response to BCR stimulation[4,5], but the proliferative response of Bcl-x_L transgenic PI3K$^{-/-}$ B cells was similar to that of WT B cells (**Fig. 3b**). Consistent with these results, transgenic expression of Bcl-x_L increased the expression of cyclin D2 in PI3K$^{-/-}$ B cells (**Fig. 3c**). These results indicate that the lack of Bcl-x_L induction is a common defect in PI3K$^{-/-}$ and Btk$^{-/-}$ B cells leading to the similar phenotypes seen in PI3K$^{-/-}$ and Btk$^{-/-}$ mice. On the other hand, T cell-independent antibody production in response to dinitrophenyl (DNP)-Ficoll, which is impaired in PI3K$^{-/-}$ mice[4], was not restored by introduction of the Bcl-x_L transgene (**Fig. 3d**), indicating that the expression of Bcl-x_L alone is insufficient for the restoration of some of the functional defects observed in PI3K$^{-/-}$ mice.

Table 2. Restoration of splenic B cell numbers in PI3K$^{-/-}$ mice by Bcl-x_L expression

Genotype[a]	No. of B cells ($\times 10^6$)	No. of IgM^{low} B cells ($\times 10^6$)	No. of T cells ($\times 10^6$)	B/T cell ratio
WT (n = 6)	34.2 ± 9.1	17.3 ± 5.3	27.1 ± 9.9	1.32 ± 0.32
Bcl-x_L tg (n = 4)	56.4 ± 16.7[b]	28.7 ± 9.3[b]	29.5 ± 9.2	1.99 ± 0.44
PI3K$^{-/-}$ (n = 7)	21.0 ± 6.7[b,c]	6.3 ± 2.2[c,e]	35.6 ± 3.2	0.60 ± 0.15[c,f]
PI3K$^{-/-}$ × Bcl-x_L tg (n = 7)	48.2 ± 10.0[d]	14.3 ± 4.6[d,b]	36.1 ± 11.2	1.42 ± 0.43[b]

[a]Mice are on a C57BL/6 background. Significance examined by Student-Newman-Keuls test: [b]$P < 0.05$ from WT; [c]$P < 0.01$ from Bcl-x_L tg; [d]$P < 0.01$ from PI3K$^{-/-}$; [e]$P < 0.05$ from WT; [f]$P < 0.01$ from WT; [g]$P < 0.01$ from Bcl-x_L tg; [h]$P < 0.05$ from PI3K$^{-/-}$; [i]$P < 0.01$ from WT; [j]$P < 0.01$ from Bcl-x_L tg; [k]$P < 0.01$ from PI3K$^{-/-}$.

Discussion

Contrary to the current model, in which PI3K acts directly upstream of Btk, tyrosine phosphorylation and subsequent activation of kinase activity of Btk was unaffected by the lack of PI3K or by PI3K inhibitors. There have been a few hints previously that this might be the case. BCR-induced Btk activation is blocked only marginally by wortmannin at 50 nM in the B cell line J558Lμm3[37]. Overexpression of the p110 catalytic subunit of PI3K in fibroblasts as well as in the B cell line A20B results in tyrosine phosphorylation of Btk. In this case as well, tyrosine phosphorylation of Btk is only modestly blocked by wortmannin, even at 100 nM, implying the presence of a PI3K-independent pathway for Btk activation[21]. A recent study further shows that tyrosine phosphorylation of Btk is unaffected in B cells deficient for p110δ, the most abundantly expressed catalytic isoform of class I_A PI3K[38]. Phenotypes of PI3K$^{-/-}$Btk$^{-/-}$ double-deficient mice were consistent with these observations. We repeatedly observed higher amounts of tyrosine phosphorylation of Btk in PI3K$^{-/-}$ B cells than in WT B cells. Likewise, PI3K$^{-/-}$ B cells showed higher kinase activity than WT B cells. The reason for the hyperactivation of Btk in PI3K$^{-/-}$ B cells is unknown at present.

The fact that a point mutation within the PH domain of Btk (in which an arginine residue critical for the binding to PIP_3 is replaced by cysteine) leads to *Xid* also supports the current model[12,15,16]. In the DT40 chicken B cell system, targeted disruption of Btk results in impaired activation of phospholipase C-γ2, which is restored by transfection of WT Btk, but not Btk with the *Xid* mutation[39]. In our hands, however, the mutant Btk protein produced from the gene carrying the *Xid* mutation was unstable and degraded rapidly when expressed in cells by gene transfer (data not shown). It is possible that the defect caused by the *Xid* mutation is not due to the inability to bind PIP_3, but to the degradation of the mutant protein. It was theoretically possible that Btk functions upstream of PI3K, but the fact that PI3K was activated in the absence of Btk excluded this possibility.

Recruitment of phosphorylated Btk to the plasma membrane was also unaffected by PI3K inhibitors or in PI3K$^{-/-}$ B cells. Recent studies have raised the possibility that Btk is recruited to the plasma membrane through a mechanism independent of PIP_3 generation. Identification of an adapter protein, BLNK (also known as SLP65), and its involvement in Btk activation support this alternative possibility[17,18]. In fact, BLNK is phosphorylated by Syk and provides Btk with docking sites to bring them into close proximity. Btk is then activated by tyrosine phosphorylation after binding to BLNK upon BCR stimulation. At the same time, BLNK is recruited to the plasma membrane upon BCR stimulation by binding to the BCR complex, which leads to the recruitment of Btk to the plasma membrane[40]. Involvement of such molecular mechanisms of recruiting Btk to the plasma membrane should be evaluated for a better understanding of the role of Btk in BCR signaling. Although PI3K and Btk likely function independently in B cell signal transduction pathways and have unique roles in proximal BCR signaling, we do not exclude the possibility that the interaction between PIP_3 and the PH domain is more critical for the activation of Btk, and possibly other Tec family kinases, in other cell types with different receptor systems.

BCR-mediated activation of Akt was completely blocked in the absence of PI3K. Activation of Akt is a multi-step reaction involving the generation of PIP_2 and PIP_3, which recruit Akt and PDK1, respectively, to the plasma membrane[22,23]. Although the role of Btk in Akt activation is controversial in the chicken DT40 B cell system[22,41], Akt activation, as revealed by phosphorylation of two critical residues in primary Btk$^{-/-}$ B cells, was unaffected. Thus, activation of Akt is dependent on class I_A PI3K containing p85α, but is independent of Btk in mouse primary B cells. Akt binds and activates IKK to induce degradation of IκB and activation of NF-κB[42]. Btk is also required for the activation of NF-κB in B cells[31,32]. Because activation of Akt does not depend on Btk in mouse B cells, it is likely that both Btk-dependent and Akt-dependent distinct pathways are required for activating NF-κB in B cells.

Induction of both Bcl-x_L and cyclin D2 involves NF-κB–mediated transcriptional activation. For example, overexpression of dominant-negative NF-κB inhibited CD40-mediated Bcl-x_L induction[43] and transgenic mice expressing a constitutively active, membrane-anchored Akt showed elevated activation of NF-κB and Bcl-x_L[44]. Bcl-x_L is a major anti-apoptotic protein that is induced upon BCR stimulation[45]. Consistent with these observations, PI3K$^{-/-}$ B cells and Btk$^{-/-}$ B cells showed increased apoptosis compared with WT B cells. Previously, we observed little significant difference in viability between PI3K$^{+/-}$ and PI3K$^{-/-}$ B cells upon BCR stimulation, as measured by annexin V staining[4]. However, we noted that annexin V staining is higher on B cells than on other cell types[45] and is not a sensitive method for measuring apoptotic B cells. As shown here, propidium iodide staining seems to be a better method to evaluate apoptosis in B cells. The lack of Bcl-x_L as well as cyclin D2 induction may be the cause of the phenotypic similarity between PI3K$^{-/-}$ and Btk$^{-/-}$ mice. In fact, forced expression of Bcl-x_L as a transgene restored B cell development and proliferative responses similar to what has been observed in *Xid* B cells[46]. These results also support our conclusion that the NF-κB-Bcl-x_L pathway is a

common target of PI3K- and Btk-dependent distinct signaling pathways in B cell activation. As observed in *Xid* mice, however, T cell-independent antibody production was not restored by introduction of the Bcl-x_L transgene, indicating that the expression of Bcl-x_L alone is insufficient for the restoration of some of the functional defects caused by the lack of Btk and PI3K.

Biochemical and genetic approaches revealed that class I_A PI3K and Btk constitute functionally distinct signaling pathways proximal to the membrane, but share a common downstream target, the NF-κB-Bcl-x_L pathway, in BCR-mediated signal transduction. The lack of activation of the NF-κB-Bcl-x_L pathway likely leads to the similarity of phenotypes in PI3K$^{-/-}$ and Btk$^{-/-}$ mice. The mechanisms that coordinate the PI3K-Akt and Btk pathways in the activation of NF-κB remain to be determined.

Methods

Mice. PI3K-deficient mice[34] were backcrossed to C57BL/6 or BALB/c mice for more than seven generations before intercrossing heterozygous mice[46,47]. Mice on a C57BL/6 background were used unless otherwise mentioned. Btk$^{-/y}$ mice on a (C57BL/6 × 129/Sv) mixed background were purchased from The Jackson Laboratory (Bar Harbor, ME). Because Btk is encoded on the X chromosome, Btk-deficient female and male mice have the Btk$^{-/-}$ and Btk$^{-/y}$ genotypes, respectively. Hence, we designate Btk-deficient mice as Btk$^{-/-}$ mice. PI3K$^{-/-}$Btk$^{-/-}$ double-deficient mice were generated by crossing PI3K$^{-/-}$ and Btk$^{-/-}$ mice to generate F2 mice carrying the PI3K$^{-/-}$Btk$^{-/-}$ genotype. Bcl-x_L transgenic mouse line #87 on a C57BL/6 background has been described[35,48]. In this transgenic mouse line, human Bcl-x_L protein is driven by the SV40 promoter and Eμ enhancer and is abundantly expressed in B cells. Bcl-x_L transgenic and PI3K$^{-/-}$ mice were crossed to generate PI3K$^{-/-}$ mice expressing the Bcl-x_L transgene in PI3K$^{-/-}$ B cells. All mice were maintained at Taconic (Germantown, NY) or in our animal facility under specific pathogen-free conditions. All experiments were performed in accordance with our Institutional Guidelines.

Reagents. Antibodies to cyclin D2, Erk2, Btk and Lyn were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Bcl-x_L was obtained from Transduction Laboratories (Lexington, KY). A mAb to Btk, 43-3B[49], was a generous gift from S. Tsukada (Osaka University, Osaka, Japan). Anti-p85^{PAN} was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-Akt, anti-phospho-Akt(S473) and anti-phospho-Akt(T308) were from Cell Signaling Technology (Beverly, MA). Specific antisera for p85β and p55γ have been described[3]. Anti-phosphotyrosine antibody (4G10) was a gift from T. Roberts (DFCI, Boston, MA). PI3K-specific inhibitors, wortmannin and Ly294002, were purchased from Calbiochem (La Jolla, CA).

Flow cytometric analysis. Fluoroscein isothiocyanate (FITC)-conjugated anti-mouse IgM, FITC-conjugated anti-mouse IgD, FITC-conjugated anti-CD19, phycoerythrin (PE)-conjugated anti-B220, biotinylated anti-mouse IgM and biotinylated anti-CD3 were purchased from PharMingen (San Diego, CA). Binding of biotinylated mAbs was detected with streptavidin-Red670 (GIBCO BRL, Grand Island, NY). One to two million cells were stained with designated antibodies in PBS with 2% fetal calf serum (FCS) and subjected to analysis on a FACScan using the CELLQuest program (Becton Dickinson, San Jose, CA).

Cell stimulation and immunoblotting. B cells were purified from total splenocytes using anti-B220-coated magnetic beads and AutoMACS (Miltenyi Biotech, Sunnyvale, CA). Purity of the cells was >95%. We resuspended 2–7 × 10^7 purified B cells in 1 ml of culture medium and preincubated them for 15 min at 37 °C with or without inhibitors. Cells were then stimulated with F(ab')$_2$ fragment of goat polyclonal antibody to mouse IgM (anti-IgM F(ab')$_2$, 40 μg/ml; Jackson ImmunoResearch, West Grove, PA) and incubated at 37 °C for the indicated time. Cells were collected, lysed in a lysis buffer solution (1% NP-40, 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, 50 μM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na-vanadate) and immunoprecipitated with the indicated antibodies or directly applied to SDS-PAGE and transferred to polyvinyldifluoride (PVDF) membranes. Reactive proteins were visualized with ECL Chemiluminescent substrates (NEN, Boston, MA). To examine phosphorylation of Btk in the membrane fraction, cells were lysed with 300 μl of hypotonic buffer solution (10 mM HEPES, pH 7.9, 10 mM NaF, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM benzamidine, 2 mM EGTA, 2 mM DTT, 1 mM vanadate, 1 mM PMSF, 1% aprotinin) using a Dounce homogenizer. Lysates were centrifuged at 10,000*g* for 30 s, and the supernatant was further centrifuged at 100,000*g* for 30 min to obtain S100 (supernatant) and P100 (pellet). P100 was subjected to immunoblot analysis with 4G10 and anti-Lyn.

PI3K activity. Activation-induced PI3K activity in B cells was estimated as PI3K activity among tyrosine phosphorylated proteins[3]. After BCR stimulation, cell lysates were immunoprecipitated with 4G10 and subjected to *in vitro* PI3K assay. Briefly, immunoprecipitate was incubated with phosphatidylinositol and γ-[^{32}P]ATP for 15 min at room temperature, and the chloroform extract was separated by thin-layer chromatography.

Btk activity. Splenic B cells (6 × 10^7) were stimulated with or without 20 μg/ml of anti-IgM F(ab')$_2$ at 37 °C for 3 min in the presence or absence of PI3K inhibitors, and lysed in an extraction buffer solution (20 mM Tris, pH 7.4, 2 mM EGTA, 12.5 mM β-glycerophosphate, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 2 mM DTT, 1 mM PMSF, 1 mM vanadate, 1% aprotinin) containing 0.2% Triton X-100. For immunoprecipitation, 15 μg of an anti-Btk was coupled to protein A Sepharose at 4 °C overnight. The beads were washed once with extraction buffer solution and incubated with precleared total cell lysates for 1 h at 4 °C. Subsequently, the beads were washed twice with extraction buffer solution containing 1% Triton X-100 and once with extraction buffer solution alone, followed by incubation with or without 100 μM ATP at 22 °C for 5 min. Samples were subjected to immunoblot analysis with 4G10. Or, immunocomplex was incubated with 5 μg of acid-denatured enolase as an exogenous substrate in the presence of 100 μM ATP at 22 °C for 5 min.

EMSA. Preparation of nuclear extract and EMSA were carried out as described[51,52]. Briefly, 10 μg nuclear extract was incubated with 20 fmol ^{32}P-labeled NF-κB probe (Santa Cruz). The DNA-protein complexes were resolved on a native 5% polyacrylamide gel, dried and exposed to an x-ray film for autoradiography. Identity of the band was confirmed by anti-p50 (Santa Cruz)-induced supershift (data not shown).

Cell proliferation and cell cycle analysis. Purified B cells (0.5 to 1 × 10^5/well) were treated with the indicated concentrations of anti-IgM F(ab')$_2$ in culture medium containing 2 ng/ml rIL-4 (Pepro Tech $EC_{1/10}$, London, England) in 96-well plates for 72 h. [^{3}H]Thymidine (3.7 × 10^4 Bq (1 μCi)/well) was added to the cultures during the last 16 h and uptake of radioactivity was measured by liquid scintillation counter. For cell cycle analysis, splenic B cells were activated with anti-IgM F(ab')$_2$ *in vitro* for 18 h, fixed with 70% ethanol and treated with RNaseA (1 mg/ml). Fixed cells were stained with 50 μg/ml propidium iodide for 3 h at room temperature and analyzed on a FACScan (Beckton Dickinson).

Antibody production. Mice were pre-bled and immunized intraperitoneally with 100 μg DNP-keyhole lympet hemocyanin (KLH; LSL, Tokyo, Japan) in a 1:1 emulsion with Freund's complete adjuvant (Sigma), or 10 μg DNP-Ficoll in PBS at day 0. The serum was analyzed at day 7 for DNP specific total immunoglobulin by ELISA.

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Competing interests statement

The authors declare that they have no competing financial interests.

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