

PI3K signaling controls cell fate at many points in B lymphocyte development and activation

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Abstract

Many receptors on diverse cell types activate phosphoinositide 3-kinase (PI3K). The lipid products of PI3K, termed 3-phosphoinositides, regulate numerous cellular processes by recruiting specific proteins to membrane signaling complexes. In the B lymphocyte lineage, PI3K activation is a critical control point at various stages of development, proliferation and differentiation. PI3K signaling is promoted by stimulatory receptors such as surface immunoglobulin, CD40, Toll-like receptors and cytokine receptors, and opposed by the inhibitory receptor Fc γ RIIB1. Genetic dissection of the PI3K pathway in mice has indicated that certain B cell functions are regulated by a limited set of PI3K isoforms and downstream effectors. Here we review our current understanding of how signals are relayed to and from PI3K in B cells. © 2004 Elsevier Ltd. All rights reserved.

Keywords: 1-Phosphatidylinositol 3-kinase; PI3K; B lymphocyte; Second messenger; Signal transduction

1. Introduction

1.1. PI3K

The phosphoinositide 3-kinase (PI3K) family of lipid kinases is so named for its role in phosphorylating the 3-hydroxyl of the inositol ring of phosphoinositides in cellular membranes. 3-Phosphoinositides play integral roles in the assembly of membrane signaling complexes and in the intracellular trafficking of proteins [1,2]. PI3K enzymes are subdivided into four classes (IA, IB, II and III) based on substrate selectivity and regulation. The Class IA and IB PI3K isoforms are the only enzymes able to produce the critical second messenger PtdIns(3,4,5)P₃ and are the focus of this review. For detailed reviews of PI3K structure and function, see [1,3].

Class IA and IB isoforms exist as heterodimers consisting of a catalytic and a regulatory subunit (Fig. 1). Interactions of the regulatory subunit with various partner proteins influence the localization and activity of the catalytic subunit. A direct interaction of the catalytic subunit with Ras can also stimulate the activity of class I PI3Ks. In mammals, three class IA catalytic isoforms exist and are termed p110 α , p110 β and p110 δ (encoded by *Pik3ca*, *Pik3cb* and *Pik3cd* genes). There are five class IA regulatory subunits encoded

by three genes: alternative splicing of the *Pik3r1* gene yields p85 α , p55 α , and p50 α , whereas p85 β and p55 γ are unique products of the *Pik3r2* and *Pik3r3* genes (Fig. 1). These regulatory subunits bind interchangeably with the catalytic subunits mentioned above. Class IA regulatory subunits each possess two highly conserved Src homology-2 (SH2) domains, which bind specifically to phosphorylated tyrosine in the specific context YXXM (where X is any amino acid). These SH2 domains are separated by a coiled-coil motif that comprises the p110-binding site. While it is likely that interactions between the SH2 domains and phosphorylated tyrosines are critical for the recruitment of class IA PI3K to tyrosine phosphorylated signaling complexes, other modular domains can contribute to PI3K regulation and may confer additional specificity among isoforms. In particular, the longer isoforms p85 α and p85 β contain additional domains in the N-terminal portion: one Src homology-3 (SH3) domain, and two proline-rich regions that flank a breakpoint cluster region homology (BH) domain (Fig. 1). The BH domain is structurally similar to GTPase activating proteins (GAPs) for Rho family small G proteins [4], and can interact with members of the Rac and cdc42 subfamilies [5], but lacks RhoGAP activity (Fig. 1).

Class IB PI3K (also termed PI3K γ) consists of a single catalytic isoform, p110 γ , and a single regulatory isoform, p101, and has been shown to be activated downstream of G-protein coupled receptors (GPCRs). p110 γ and the class IA catalytic subunit p110 δ are expressed primarily in leukocytes, while the remaining class IA catalytic subunits p110 α

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and p110 β are ubiquitously expressed. All class I catalytic subunits contain a Ras binding domain and a C2 domain in addition to the kinase domain (Fig. 1) [6].

A number of modular protein domains have evolved to interact with specific phosphoinositides [1,2]. The pleckstrin homology (PH) domain is perhaps the best studied in the context of lymphocyte signaling. PH domains selective for 3-phosphoinositides are found in several critical signaling enzymes. These include phosphoinositide-dependent kinase-1 (PDK-1), Akt (also termed PKB), Tec family tyrosine kinases, and certain guanine nucleotide exchange factors (GEFs) and GAPs for small G proteins. Although a wide range of stimuli activate PI3K, it is thought that additional interactions engaged by distinct receptors enforce specificity in the activation of 3-phosphoinositide-binding proteins (“PI3K effectors”). For example, pREX-1, a RacGEF that functions downstream of GPCRs for chemotactic ligands, is fully activated only in the presence of both PtdIns(3,4,5)P₃ and G protein $\beta\gamma$ subunits [7,8].

PI3K signaling is opposed by lipid phosphatases. Phosphatase and tensin homolog (PTEN) is a 3-phosphatase that converts PtdIns(3,4,5)P₃ back to PtdIns(4,5)P₂, the original substrate for class I PI3K. PTEN is the product of a tumor suppressor gene that is commonly mutated in advanced cancers in humans, and PTEN ablation has dramatic

consequences for lymphocyte development and activation [9,10]. SH2-containing inositol phosphatases (SHIP1 and SHIP2) are 5-phosphatases that convert PtdIns(3,4,5)P₃ to PtdIns(3,4)P₂. SHIP1 is involved in inhibitory receptor signaling in the immune system, and deletion of SHIP1 decreases activation thresholds in several hematopoietic lineages including B cells [11].

A great variety of cell surface receptors can mediate transient increases in PtdIns(3,4,5)P₃, a hallmark of class I PI3K activation. This is usually accompanied by accumulation of PtdIns(3,4)P₂, often with delayed and sustained kinetics [12], suggesting that some of the latter lipid is produced by dephosphorylation of PtdIns(3,4,5)P₃ by 5-phosphatases. Using chromatographic methods, Gold and Aebersold were the first to demonstrate PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ production in murine B cell lines activated via the B cell receptor (BCR) [13]. Subsequently, it was shown that pharmacological inhibitors of PI3K could block proliferation of primary B cells from human [14] or mouse [15], whether induced by engagement of the BCR, CD40, Toll-like receptors (TLRs), or cytokine receptors. Using a genetically encoded fluorescent probe for PtdIns(3,4,5)P₃, Astoul et al. were able to demonstrate plasma membrane accumulation of PtdIns(3,4,5)P₃ following BCR crosslinking of A20 murine B lymphoma cells, which could be blocked by the PI3K

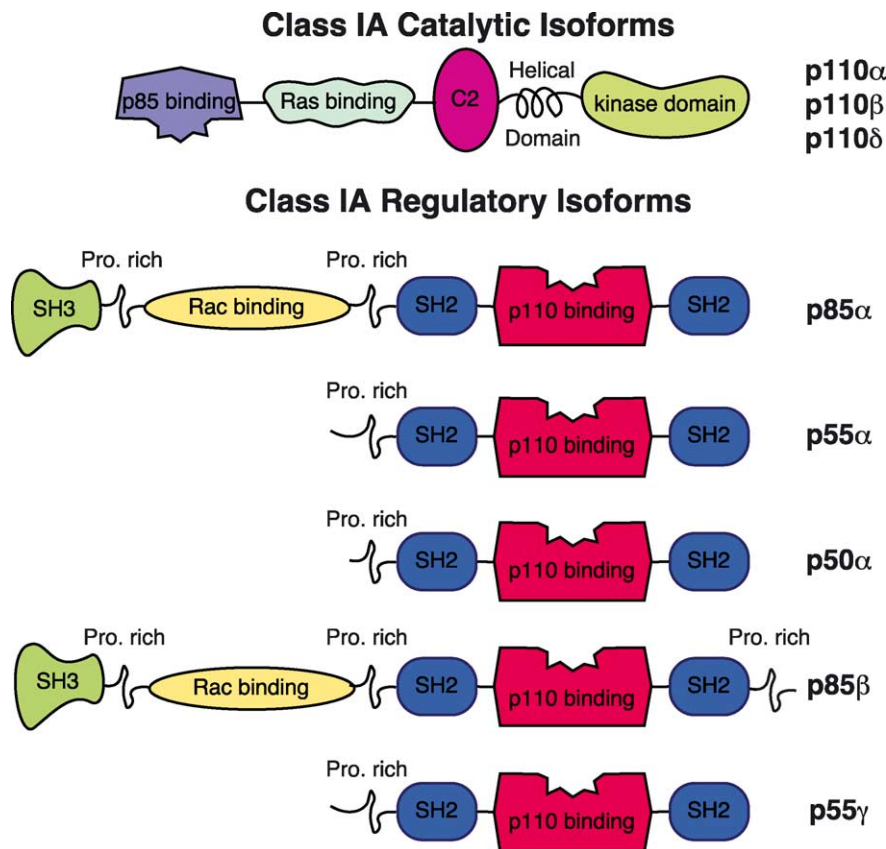


Fig. 1. Domain structure of mammalian class IA PI3Ks. The five regulatory isoforms of class IA associate interchangeably with the three catalytic isoforms, and are encoded by three genes; p85 α , p55 α , and p50 α are alternative transcripts of a single gene.

inhibitor LY294002 [16]. Accumulation was also blocked by co-ligation of the inhibitory receptor FcγRIIB1, presumably through its recruitment of SHIP1. The fluorescent probe in these experiments consisted of GFP fused to the PH domain of Akt, the specificity of which allowed the fusion protein to bind to PtdIns(3,4,5)P₃ at the membrane [16]. The same group has generated transgenic mice expressing this probe in T cells, and used the mice for elegant studies of the dynamics of PtdIns(3,4,5)P₃ accumulation at the immunological synapse [17]; similar studies in primary B cells have not yet been reported.

1.2. B cell development and activation

The classification of developing B cells into discrete subsets has been a topic of active research for many years [18,19]. Although there is still considerable debate about details of B cell development and lineage commitment, an overall outline of B cell ontogeny can be summarized as follows (Fig. 2). The earliest committed B cell progenitors in the bone marrow are termed pro-B cells. At this stage the immunoglobulin (Ig) heavy chain loci undergo gene rearrangement, and those cells that successfully produce a functional heavy chain express a “pre-B cell receptor” on the

surface consisting of the heavy chain, surrogate light chains, and the signaling chains Ig-α and Ig-β. Assembly of the pre-BCR allows transition to the pre-B cell stage, at which time light chain gene rearrangement occurs. Cells that express a functional light chain/heavy chain in association with Ig-α and Ig-β are classified as immature B cells because engagement of this BCR complex triggers apoptosis or anergy rather than proliferation. This allows for induction of tolerance to self-antigens before export of mature B cells. B cells exit the bone marrow as “transitional” B cells and take up residence in the spleen, where they can still be subject to tolerance induction. After various substages as transitional B cells, surviving cells enter the mature, recirculating pool that can respond to antigen.

Mature B cells in the mouse are generally categorized into two subsets: B1, which reside mainly in body cavities like the peritoneum, and B2, which are found in the lymphoid organs and blood (Fig. 2). B2 cells are further subdivided into follicular (FO) B cells and marginal zone (MZ) B cells. FO B cells are migratory cells that circulate through the periphery and lymphoid organs, and upon encountering antigen, proliferate in the lymphoid follicles and germinal centers to produce high affinity antibody and immunological memory. MZ B cells are sessile cells in the spleen marginal zone, and

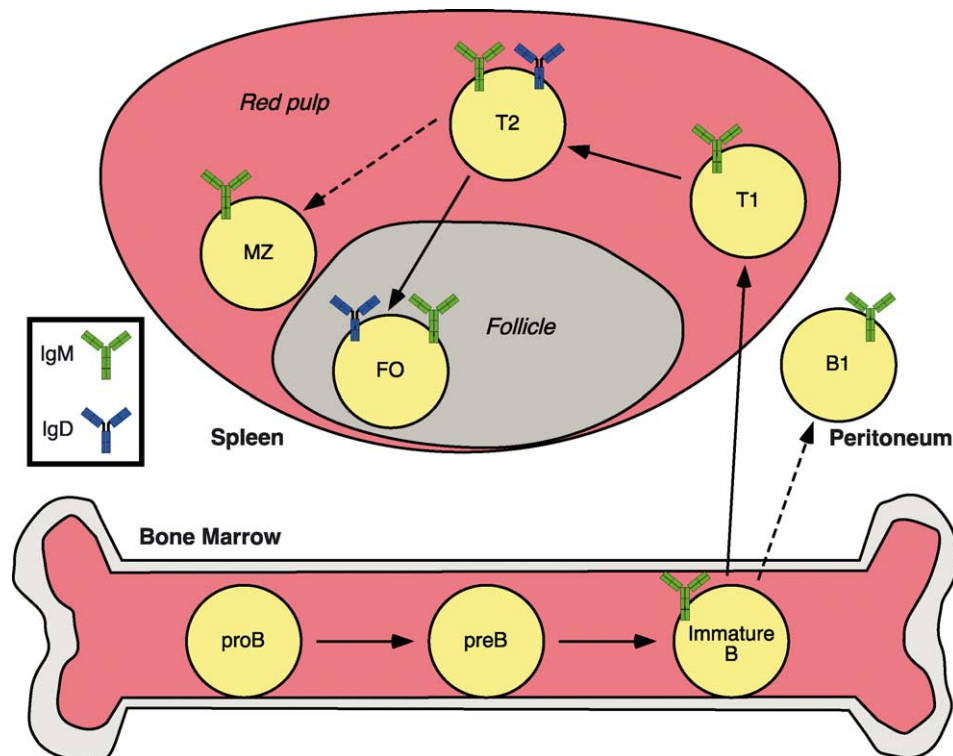


Fig. 2. Localization and surface Ig expression of B cell developmental subsets. Common lymphoid progenitors give rise to pro-B cells in the bone marrow, which pass through a pre-B cell stage before becoming IgM isotype-expressing immature cells. Immature B cells exit the bone marrow and migrate to the spleen, becoming the B2 subset. The origin of the B1 subset found in the peritoneum is debated (hatched arrow). Maturation continues in the spleen, where immature cells pass through two transitional stages: T1, which express only IgM, and T2, which express both IgM and IgD. T2 cells then give rise to mature, recirculating B cells, which are found in the follicle of the spleen and are therefore referred to as follicular cells (FO). The surface expression of antigen receptors on FO cells is that of the classic mature B cell, IgDh/IgMl. The phenotypically distinct B cells known as marginal zone (MZ) cells encircle the splenic follicle, and express higher levels of IgM and little IgD; the developmental origin of MZ B cells is debated (hatched arrow).

like the B1 subset, may have specialized functions including the production of “natural” antibodies and rapid responses to common pathogens.

Productive B cell activation and antibody secretion generally requires a second signal in addition to BCR engagement. This can be provided by TLR ligands such as lipopolysaccharide (LPS), or by activated T cells that express CD40 ligand (CD40L) and secrete cytokines such as IL-4. In vitro, B cell proliferation can be triggered in the absence of BCR engagement by high concentrations of TLR ligands, or by the combination of CD40 ligation and IL-4.

2. Evidence for PI3K function at decision points in B cell lineage

Most cell fate decisions in B cells are regulated by signaling through the BCR or pre-BCR itself, but are also influenced by cell surface structures and cytokines derived from stromal cells and other lymphocytes in the bone marrow and spleen. Given that PI3K is activated following engagement of the BCR, CD40, and numerous cytokine receptors, as well as by TLR-mediated signals, one might predict that PI3K function is required at multiple points in the lifetime of a B cell. This hypothesis is supported by a growing body of literature demonstrating that PI3K regulates developmental steps in bone marrow precursors, differentiation of mature B cell subsets, activation and proliferation of mature B cells, and generation of antibody responses in vivo.

Genetic evidence for this hypothesis has been provided by the similar developmental phenotypes of mice deficient for two PI3K genes highly expressed in lymphocytes: the p85 α regulatory isoform or the p110 δ catalytic isoform (Table 1). Loss of p85 α alone, or of all three variants of the *Pik3r1* gene, leads to a partial block at the pro-B stage, a reduction in the total number of splenocytes, and a near absence of the B1 subset [15,20]. Similar patterns were reported for three different mouse lines lacking p110 δ [21–23]. Further, all p110 δ -deficient mice generated were found to have severe reductions in MZ cells. Interestingly, similar defects in B cell development are found in mice lacking the B cell co-receptor

CD19, an important activator of PI3K [24,25]. Mice lacking p85 α alone have a partial reduction in MZ B cells (A.C.D. and D.A.F., unpublished data). A role for PI3K activity in B cell development is further strengthened by the finding that B lineage-specific deletion of PTEN, the direct antagonist of PI3K function, leads to augmented numbers of MZ and B1 cells, and rescues the defect found in CD19-deficient mice [26].

Genetic models have also supported a role for PI3K in proliferation and/or survival of splenic B cells, following stimulation via the BCR, TLRs, CD40 or the cytokine IL-4. BCR-mediated proliferation is almost completely abolished in B cells isolated from mice deficient for either p85 α or p110 δ [15,20–23]. In addition, proliferation triggered by LPS, a TLR4 agonist, or by CD40 crosslinking, is partially impaired in these mice. Conversely, proliferation driven by these agonists is enhanced in B cells lacking PTEN [26,27]. IL-4 alone is mainly a survival factor for primary B cells but enhances the proliferation mediated by the BCR; both of these responses are diminished in cells treated with PI3K inhibitors or lacking p85 α or p110 δ [15,20–23]. There is disagreement about whether loss of PTEN protects against apoptosis in B cells. One group found increased survival, as expected [27], while another found increased death [26], which they attributed to inappropriate cell cycle entry.

One caveat to interpreting data from these genetic models is the fact that splenic B cell preparations from knockout mice have different ratios of immature and mature subsets that may have different activation properties. However, further support for the importance of PI3K in BCR-mediated proliferation is derived from studies of inhibitory receptor signaling (Fig. 3). The inhibitory receptor Fc γ RIIB1 plays an important role in limiting B cell activation late in immune responses. Antigens that are already coated with specific IgG cause co-clustering of the BCR with Fc γ RIIB1, triggering the Fc receptor to send an inhibitory signal to prevent productive B cell activation. This phenomenon has been studied in vitro by comparing B cells stimulated with F(ab')₂ fragments of anti-Ig (BCR engagement only) to cells stimulated with intact anti-Ig (BCR plus Fc γ RIIB1). It is now clear that a central mechanism of inhibitory signaling is the

Table 1

Summary of knockout phenotypes for PI3K isoforms and selected phosphoinositide phosphatases (references in italics)

Gene	Category	Defects in:		
		Development	Activation	Ab production
p110 δ [21–23]	Class IA catalytic	Decreased FO; loss of MZ, B1	α IgM, LPS, α CD40, IL-4	TI and TD
p110 γ [131]	Class IB catalytic	Changes in light chain ratios	None	None
p85 α [20]	Class IA regulatory	Decreased FO, MZ ^a ; loss of B1	α IgM, LPS, α CD40, IL-4	TI-2
p85 α /p55 α /p50 α [15]	Class IA regulatory	Decrease in mature cells; loss of B1	α IgM, LPS, α CD40, IL-4	N.D.
p85 β 113	Class IA regulatory	None	None	None
PTEN [10,26,27]	3-Phosphatase	Increased MZ; increased B1	Increased proliferation	Increased basal IgM; impaired CSR
SHIP [11,29,30]	5-Phosphatase	Faster transitions	Lower thresholds	Increased basal Ig

^a A.C.D. and D.A.F., unpublished analysis of MZ B cells in p85 α KO mice.

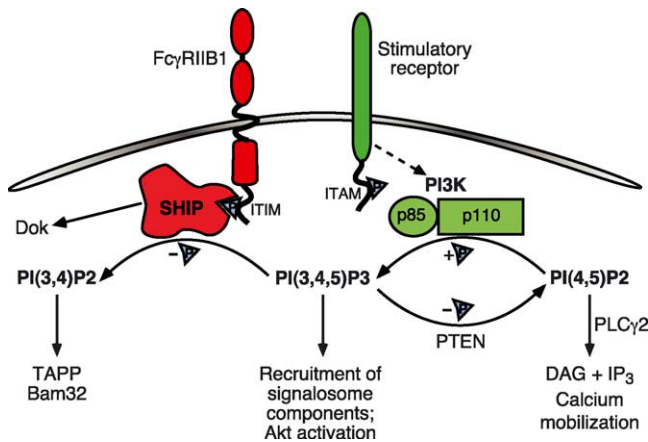


Fig. 3. Schematic diagram of phosphoinositide modification downstream of stimulatory and inhibitory receptors. Crosslinking of stimulatory receptors (i.e. the Ag receptor) on B cells leads to the activation of PI3K and the generation of PtdIns(3,4,5)P₃ from PtdIns(4,5)P₂, thereby recruiting PH domain-containing signaling molecules to the membrane and activating Akt. The catalytic function of PI3K is directly opposed by the action of the 3-phosphatase PTEN, which converts PtdIns(3,4,5)P₃ back to PtdIns(4,5)P₂, the PI3K and PLCγ2 substrate. Cleavage of PtdIns(4,5)P₂ by PLCγ2 produces the second messengers DAG and IP₃ and initiates Ca²⁺ mobilization. Ligation of the B cell inhibitory receptor, FcγRIIB1, results in the phosphorylation of tyrosine residues in the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the cytoplasmic tail of the receptor, providing binding sites for the SH2 domain of the 5-phosphatase SHIP. Active SHIP removes the 5-phosphate from PtdIns(3,4,5)P₃, yielding PtdIns(3,4)P₂, which may recruit a distinct set of PH domain-containing proteins including Bam32. Metabolism of PtdIns(3,4,5)P₃ is not the only functional consequence of SHIP recruitment by FcγRIIB1; SHIP also opposes Ras signaling via activation of Dok. Blue triangles represent phosphate groups; hatched arrows in this and subsequent figures represent steps omitted for simplicity. ITAM, immunoreceptor tyrosine-based activation motif.

recruitment of SHIP1 to the cytoplasmic tail of FcγRIIB1 and subsequent metabolism of PtdIns(3,4,5)P₃ (reviewed in [28]). B cells lacking FcγRIIB1 or SHIP1 are unaffected by treatment with intact anti-Ig. Interestingly, SHIP1 also appears to regulate PI3K signaling in a manner that may be independent of its association with inhibitory receptors. SHIP1 deletion increases the rate at which developing B cells progress through the immature and transitional stages, and enhances survival and proliferation following engagement of the BCR alone, CD40 or TLR4 [29].

The primary job of B cells in the immune response is to produce antibodies. B cell activation by the various mitogens mentioned above is followed by differentiation into antibody-secreting plasma cells, often after cells have undergone immunoglobulin class switching and somatic hypermutation to refine the antibody response. Antibody secretion by human B cells *in vitro* is almost completely abrogated by PI3K inhibitors [14], even under conditions where proliferation is partially maintained. In mice, loss of p85α or p110δ results in a dramatic block in antibody responses to T cell-independent (TI) antigens (i.e. polymeric substances that induce massive BCR crosslinking)

[20–23]. The response to T-dependent (TD) antigens (i.e. protein antigens that are processed and presented to T cells to receive help for B cell activation) is unimpaired in p85α-deficient mice, but is reduced in mice lacking p110δ. Interestingly, proliferation induced by anti-CD40 plus IL-4, an *in vitro* mimic of T cell-dependent B cell activation, is also partially impaired in mice lacking p110δ but not p85α [15,21–23]. In chimeric mice containing SHIP1-deficient B cells, basal levels of serum Ig are increased [30]. Likewise, in mice with PTEN-deficient B cells there is enhanced basal IgM [27]. However, this is at least in part the result of impaired immunoglobulin class switching, correlating with a failure of PTEN-deficient B cells to upregulate activation-induced cytidine deaminase (AID), an enzyme required for initiation of this process [27].

Calcium (Ca²⁺) is a critical second messenger in a variety of cellular processes and is particularly important for lymphocyte activation triggered by antigen receptors [31]. In B cells, BCR engagement is followed by a rapid and sustained rise in intracellular Ca²⁺ that is partially dependent on PI3K activation. Co-ligation of FcγRIIB1 attenuates Ca²⁺ mobilization, as does pretreatment of cells with the PI3K inhibitors wortmannin or LY294002 (Fig. 4) [32]. Ca²⁺ mobilization is also diminished in p110δ-deficient B cells [22,23], and in p85α-deficient B cells (A.C.D. and D.A.F., unpublished data). Conversely, SHIP knockout B cells show an increase in the amplitude of the Ca²⁺ flux following stimulation with intact anti-Ig [30,33]. The mechanism by which PI3K contributes to Ca²⁺ mobilization is an active area of research and is introduced in the following section.

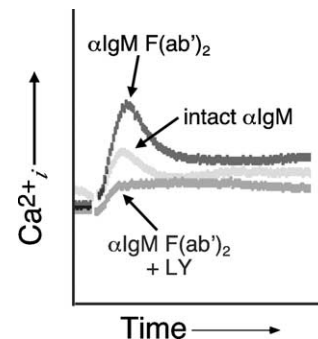


Fig. 4. Example of an experiment showing that Ca²⁺ flux is inhibited by loss of PI3K function and by ligation of the inhibitory receptor FcγRIIB1. B cells loaded with the calcium indicator dye indo 1-AM were pretreated with the pan-PI3K inhibitor LY294002 or with ethanol diluent for 15 min, and stimulated with either the F(ab')₂ fragment of anti-IgM (αIgM F(ab')₂) to crosslink the BCR alone, or with intact anti-IgM antibody to engage both the inhibitory and stimulatory receptors. Traces shown represent internal Ca²⁺ levels (Ca_i²⁺) derived from the ratio between Ca²⁺-bound and Ca²⁺-free indo-1, which fluoresce at different wavelengths. Co-ligation of the inhibitory receptor (intact αIgM) blunts the peak representing Ca²⁺ release from internal stores, and leads to a lower level of sustained Ca²⁺ influx; global inhibition of PI3K activity with LY294002 (αIgM F(ab')₂ + LY) leads to an even greater defect in both the peak and sustained phases of Ca²⁺ flux. These data support a role for PI3K in the generation of the Ca²⁺ signal downstream of the antigen receptor.

3. The signalosome model

The initial spike in intracellular Ca^{2+} following BCR crosslinking is triggered by release of the ion from intracellular stores. This is followed by opening of “store-operated” channels in the plasma membrane, with subsequent Ca^{2+} entry mediating a sustained period of calcium oscillations that maintain the average Ca^{2+} concentration above baseline [31,34]. The Ca^{2+} response is initiated by phospholipase C-gamma ($\text{PLC}\gamma$)-mediated hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ to generate diacylglycerol (DAG) and IP_3 , the latter a second messenger responsible for Ca^{2+} release from intracellular stores. A model has emerged in recent years in which $\text{PLC}\gamma$ activation downstream of the BCR is achieved by a group of signaling enzymes and scaffolding proteins that associate in a “signalosome” [35,36]. This model, which will be discussed at various points in this review, posits that 3-phosphoinositides function to promote assembly of the signalosome components at the B cell membrane, in proximity to the BCR and the substrate for $\text{PLC}\gamma$.

A current working model of the signalosome is shown in Fig. 5A. Critical components include the cytoplasmic tails of $\text{Ig}\alpha$, $\text{Ig}\beta$ and CD19, tyrosine kinases of the Src and Syk families, adapter scaffolding proteins BLNK and BCAP, members of the Vav family of RacGEFs, Rac family

small GTPases, PI3K regulatory and catalytic subunits, the 3-phosphoinositide products of PI3K, the Tec family kinase Btk, and $\text{PLC}\gamma 2$, which is the predominant $\text{PLC}\gamma$ isoform in B cells. Mice lacking any of the listed protein components show defects in B cell development and/or proliferation, and in nearly all cases examined there is a blunted Ca^{2+} mobilization response following BCR crosslinking [15,20–23,25,37–50]. Importantly, the human immunodeficiency X-linked agammaglobulinemia (XLA) is caused by mutations in Btk and a similar syndrome is seen in rare patients with homozygous loss of function in BLNK [51,52].

These genetic correlations suggest that these gene products work in a common pathway. In addition, a wealth of biochemical evidence has accumulated to support direct physical interactions among signalosome components [35,36,53,54]. Src family kinases and Syk associate with Ig receptor tails and can phosphorylate critical tyrosine residues on CD19, BCAP and BLNK to generate docking sites for SH2 domains of other signalosome components. Of particular importance, CD19 phosphorylation leads to the binding of class IA PI3K regulatory isoforms and Vav, and BLNK phosphorylation leads to association with Btk and $\text{PLC}\gamma 2$. Rac can be brought to the complex and activated via Vav proteins, and Rac itself can interact with PI3K via the BH domain of $\text{p}85\alpha$. Both Btk and $\text{PLC}\gamma 2$ possess domains that

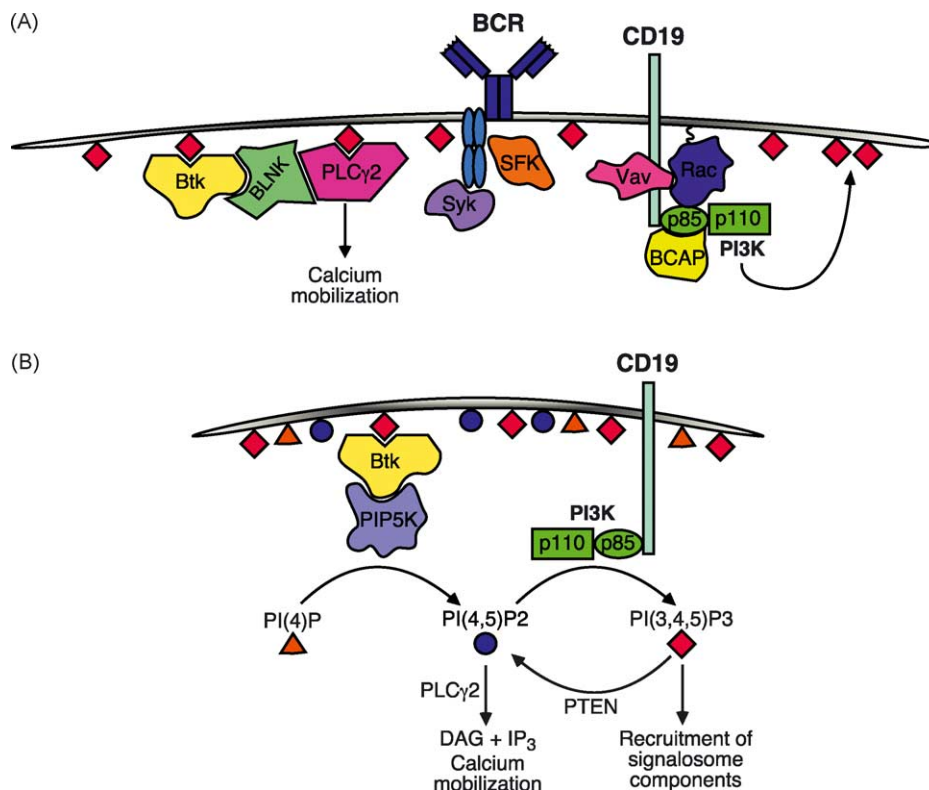


Fig. 5. Phosphoinositides in B cell signaling. Blue circles represent $\text{PtdIns}(4,5)\text{P}_2$ and orange triangles represent $\text{PtdIns}(4)\text{P}$ in this figure; red diamonds represent $\text{PtdIns}(3,4,5)\text{P}_3$ in this and subsequent figures. (A) Schematic diagram of the signalosome model of BCR signaling. $\text{PtdIns}(3,4,5)\text{P}_3$ is important for assembling signalosome components at the plasma membrane, as described in the text. Activation of $\text{PLC}\gamma 2$ by Btk and Syk leads to Ca^{2+} mobilization. SFK = Src family kinase. (B) Proposed role of Btk in maintenance of local $\text{PtdIns}(4,5)\text{P}_2$ concentrations. Recent evidence indicates that Btk binds to and shuttles to the membrane $\text{PtdIns}(4)\text{P}$ -5-kinase (PIP5K), the enzyme that generates $\text{PtdIns}(4,5)\text{P}_2$, the substrate of both PI3K and $\text{PLC}\gamma 2$.

selectively bind to PtdIns(3,4,5)P₃ and their enzyme activities, at least in vitro, are enhanced in the presence of this lipid. Local production of PtdIns(4,5)P₂, the substrate for both PLC γ 2 and PI3K, can be enhanced by recruitment of PtdIns(4)P-5-kinase (PIP5K) by either Rac [5], Vav [55], or Btk [56]. Finally, critical tyrosines in PLC γ 2 can be phosphorylated by Syk and Btk.

Although a common null phenotype could be consistent with a linear model of signal transduction, this complex web of interactions tends to support the idea that these components work together in ways that cannot always be easily defined as temporally “upstream” and “downstream”. In this model, two essential scaffolds are the protein BLNK and the phosphoinositide PtdIns(3,4,5)P₃. These components appear to be critical for recruitment of PLC γ 2 to the membrane, where its substrate is located and where the phospholipase can receive inputs from other signalosome components to achieve full activity. PLC γ activity is promoted in part by tyrosine phosphorylation mediated by Syk and Btk [37,57,58], the latter of which is itself recruited to the membrane by BLNK and PtdIns(3,4,5)P₃ [59,60]. The importance of the binding of the Btk PH domain to PtdIns(3,4,5)P₃ is illustrated by *Xid* mice. *Xid*, for X-linked immunodeficiency, arises from a single, naturally occurring point mutation (R28C) in the PH domain of Btk that abolishes selective binding to PtdIns(3,4,5)P₃ [51]. Even though the kinase is intact and other modular domains are unaltered, the *Xid* phenotype is nearly indistinguishable from that of Btk null mice [38]. Notably, similar Btk-PH domain mutations are found in some humans with XLA.

One of the issues that confront the signalosome model is the question of how the signaling complex is localized to the vicinity of the BCR, Src family kinases and Syk. Glycolipid-enriched membrane microdomains (GEMMs), or lipid rafts, are likely to play an important role in this process. In unstimulated cells, rafts are enriched in signaling proteins that have specific lipid modifications, in particular Src family kinases. Recent evidence indicates that BCR crosslinking is followed by movement of the BCR into rafts, along with formation of raft aggregates. Signalosome components reported to partition to lipid rafts include BLNK [64] and PLC γ 2 [61], while Lyn is constitutively associated with GEMMs [62]. PI3K was shown to localize to lipid rafts in DT40 cells [63], and ligation of the pre-BCR in human cells leads to the recruitment of all of these signalosome components, as well as Syk and Vav [64]. Nucleation of the signalosome in rafts may be mediated in part by the newly discovered linker of activated B cells (LAB [65]), an adapter protein that appears to be structurally and functionally equivalent to linker of activated T cells (LAT).

Might PI3K also regulate signalosome assembly directly via effects on raft aggregation? This is suggested by the observation that co-ligation of Fc γ RIIB1 leads to the disruption of rafts in mature B cells. As yet, there have been no reports of the effect of PI3K inhibitors or gene knockouts on the stability of lipid rafts in B cells (though one group

found that deletion of p110 δ decreased raft aggregation in stimulated T cells [22]). Conversely, there is no clear evidence that destabilization of rafts affects the magnitude or kinetics of PI3K activation. Early studies using chemical destabilization of rafts are confounded by later work showing that these reagents are non-specific.

The components of the signalosome have been defined by a combination of biochemical and genetic evidence. It is important to keep in mind the limitations of each of these approaches. Many biochemical assays are difficult to perform on primary B cells; therefore, much of the existing evidence for protein–protein and protein–lipid interactions has been derived from in vitro measurements or immortalized cell lines that can often be poor models of physiological signaling. Interpretation of studies using primary splenic B cells is complicated by the heterogeneity inherent in the cell preparation. It is now established that distinct subsets of immature and mature B cells have different signaling thresholds and functional responsiveness. This is a particular problem when comparing wild-type cells to cells from mutant mice, in which the abundance of immature and mature B cell subsets is altered. Such changes may mask or magnify effects generated by the genetic changes made. For example, defects in LPS-mediated proliferation of B cells lacking p110 δ could be attributable to the absence of MZ B cells, the subset most sensitive to this mitogen. Ultimately, assays capable of analyzing signaling and function in individual subsets are required to strictly test whether different mutations produce a similar phenotype. This type of analysis has been used to show specific defects in transitional splenic B cells in Btk-deficient mice [66].

It is worth noting that detailed investigation of development and signaling is beginning to reveal differences among mice lacking putative signalosome components. For example, CD19-deficient and p110 δ -deficient mice lack MZ B cells whereas Btk-deficient mice do not [18,22,23,25,67]. In addition, the Ca²⁺ mobilization response is less severely impaired in B cells lacking Btk than in cells lacking several other components. These findings do not negate the likely role of Btk in the signalosome, because mouse B cells express the tyrosine kinase Tec that is partially redundant with Btk [68]. However, it is also worth considering that the composition of the signalosome may vary during development or that different signalosome components have distinct quantitative inputs to the activation of PLC γ 2. Finally, some components may function outside the signalosome to initiate distinct signals, as described for PI3K in Section 5.

4. Mechanisms of PI3K activation

PI3K function has been documented in many cell types, downstream of several different types of receptors, most of which use distinct mechanisms to activate the lipid kinase. This is well illustrated by studies of B cell signaling. PI3K activation is achieved by different mechanisms downstream

of the BCR, CD40, TLRs and IL-4. In each case, phosphotyrosine (pTyr) binding by SH2 domains within class IA regulatory subunits appears to play a role; however, the pTyr-containing proteins and other contributing interactions are distinct.

In the case of the BCR, there is strong evidence that Syk activity is required for PI3K activation. Deletion of Syk in the chicken B cell line DT40, or expression of kinase-deleted Syk in murine A20 cells, impairs BCR-stimulated production of PtdIns(3,4,5)P₃ [69]. Syk can phosphorylate many proteins in the signalosome (Fig. 5A), at least two of which (CD19 and BCAP) possess tyrosines in the optimal context for PI3K binding. CD19-deficient B cells have reduced PI3K activation as evidenced by a decrease in BCR-mediated phosphorylation of Akt (a commonly used readout for PI3K activity) [70]. The reduction in Akt phosphorylation is not complete, however, suggesting that other interactions contribute to PI3K activation. In DT40 chicken B cells, BCAP is required for optimal PI3K activation; however, PI3K function is apparently intact in murine B cells lacking BCAP [50,71]. B cells lacking both CD19 and BCAP have not yet been studied.

Activation of PI3K appears to be essential for CD19 signaling function. When an altered form of CD19 that lacks critical tyrosines required for PI3K binding was expressed in CD19-deficient mice, the knockout phenotype was not rescued [72]. CD19 lacking other tyrosines, including those involved in recruitment of Src family kinases and Vav, could restore function. Notably, deletion of PTEN restores the B1 and MZ B cell compartments in CD19-deficient B cells [26].

There is growing evidence that the small G protein Rac contributes to PI3K activation in several immune receptor contexts. In BCR signaling, initial evidence was provided by a study of DT40 chicken B cells in which expression of dominant negative Rac, or deletion of Vav3, an exchange factor (GEF) for Rac, diminished PI3K activation and Ca²⁺ flux [63]. Interestingly, deletion of SHIP1 complemented the defect in Ca²⁺ signaling, suggesting that Vav and Rac contribute to signalosome function primarily through enhancement of PI3K activation. More recently, Akt activation was shown to be impaired in murine B cells lacking Rac2 and one allele of Rac1 [49]. In vitro, GTP-bound Rac or the related G protein cdc42 can stimulate class IA PI3K activity [63,73]. This activation may occur via binding of Rac-GTP to the BH domain of p85 α and possibly p85 β , which have homology to RacGAPs but lack GAP activity. In addition to the apparent direct enhancement of PI3K enzyme activity, this mechanism could serve other important functions: first, to allow both PI3K and other effectors to be recruited simultaneously to this site, most notably PIP5K; second, to compete with bona fide RacGAP proteins and maintain Rac in the active state. Another possibility, to localize PI3K to a particular membrane location where Rac is enriched, is contradicted by the finding that recruitment of PI3K to rafts is not impaired in Vav3-deficient DT40 cells [63]. It should be noted that earlier models placed Vav downstream of PI3K

owing to the presence of a PH domain in Vav proteins [74]; however, this model has not been substantiated in immune signaling systems [93].

Gab1, one of a family of adaptor proteins that also includes Gab2 and Gab3, may function in a positive feedback loop in PI3K signaling [75]. Gab proteins are able to bind PtdIns(3,4,5)P₃ by virtue of specific PH domains, and following recruitment to the membrane, they are tyrosine phosphorylated on specific residues which provide binding sites for additional PI3K molecules. It has been established that Gab2 functions as a PI3K response amplifier in the context of Fc receptor signaling systems in mast cells and macrophages [76,77]. Supporting a similar model in B cells, overexpression of Gab1 in WEHI-231, an immature murine B cell line, increased activation of Akt downstream of the BCR [78]. However, mice with Gab1-deficient B cells did not show functional impairments and demonstrated increased antibody production in response to T-independent type-2 antigens [79]. This indicates a possible negative regulatory role for Gab1, though further biochemical studies of these cells are needed to understand the mechanism. Studies of the related adaptor Gab2 in a breast cancer cell line identified a role for this protein in Akt-mediated feedback inhibition of receptor signaling, and Gab2 has also been shown to oppose antigen receptor signals in T cells [80–82]. Thus, Gab family adaptor proteins may play both positive and negative regulatory roles in cell activation.

The mechanisms by which other B cell activating receptors activate PI3K are less well studied. However, recent data indicate that CD40 recruits PI3K to a signaling complex containing Src, TRAF6, and Cbl proteins [83]. Studies of dendritic cells lacking c-Cbl and B cells lacking Cbl-b demonstrated a required role for these proteins in activation of PI3K and Akt downstream of CD40 [83]. The apparent role for Cbl proteins in promoting mitogenic signaling downstream of CD40 contrasts with the role of this family of adapter molecules in opposing activation signals initiated by the BCR and TCR.

Two mechanisms have been described by which TLR family members might activate PI3K. TLR4 engagement leads to the association of PI3K with MyD88 [84]. MyD88 contains a YXXM motif, and could provide a general mechanism for recruitment of class IA PI3K downstream of TLR family members (and the related IL-1 receptor family). A role for Rac upstream of PI3K was also shown in a study of TLR2 signaling [85].

A number of cytokines that regulate B cell fate decisions can activate PI3K. IL-7 and stem cell factor (SCF) are cytokines that drive early B cell development, and IL-4 is important for proliferation and differentiation of mature B cells. The cytoplasmic tail of IL-4R is phosphorylated by the Jak kinases, which leads to the recruitment of IRS-1/2 proteins [86]. These proteins are subsequently phosphorylated at several tyrosines in the YXXM consensus, allowing class IA PI3K to bind via the SH2 domains of regulatory subunits. IL-7R is able to activate PI3K in a similar manner

as IL-4R following receptor crosslinking, as well as by phosphorylation of a YXXM motif in the tail of the IL-7R α chain, which can bind PI3K directly [87]. The receptor for SCF, c-kit, has intrinsic tyrosine kinase activity in the cytoplasmic tail and its activation leads to autophosphorylation on tandem YXXM motifs [88,89]. While the role of specific tyrosine residues in c-kit function has been well studied in the context of mast cell function and spermatogenesis, the importance of PI3K binding for B cell development is not yet clear.

5. Effectors and downstream signals

Many signaling proteins possess modular domains that specifically recognize 3-phosphoinositides [1,2,90]. A number of these putative PI3K effectors are expressed in B cells. As mentioned above, the Tec family tyrosine kinase Btk (and Tec itself) contains a PH domain that binds PtdIns(3,4,5)P₃, and a point mutation that abrogates selective binding causes *Xid* in mice and some cases of XLA in humans. Based on these findings and the similar phenotypes of mice lacking class IA PI3K genes (p85 α or p110 δ) or lacking Btk function, it was inferred that Btk is a critical PI3K effector downstream of the BCR. However, subsequent data have indicated that other PI3K effectors play important roles in B cell activation; furthermore, the mechanism by which 3-phosphoinositides regulate Btk function appears more complicated than originally assumed.

Experiments carried out *in vitro* and in transfected cell lines have suggested that Btk is activated in two ways by PtdIns(3,4,5)P₃: directly, by relieving an inhibitory interaction of the PH domain with the kinase domain [91], and indirectly, by recruiting the enzyme to the membrane to facilitate phosphorylation of a tyrosine residue in its activation loop by Src family kinases [59]. Genetic evidence for an important link between Btk and PI3K is the finding that BCR crosslinking of p110 δ -deficient B cells results in impaired phosphorylation of the activation loop tyrosine in Btk [23]. However, this finding is in contrast to those of other groups who have reported that treatment with PI3K inhibitors, or loss of p85 α or p110 δ , does not affect Btk phosphorylation [21,92]. The use of non-specific antiphosphotyrosine antibodies in the latter reports could have masked differences in phosphorylation of critical activation loop tyrosines [93], but this explanation does not account for the apparent maintenance of Btk enzyme activation in the absence of PI3K function [92]. In addition, phosphorylation of PLC γ 2 is at least partially maintained in cells lacking p110 δ [21,23]. Furthermore, kinase-dead Btk has been shown to promote Ca²⁺ flux [56,94]. Could Btk have a kinase-independent function that is dependent on binding to 3-phosphoinositides?

This idea has gained some support from the recent finding that Btk associates with PIP5K and shuttles it to the membrane [56]. This enzyme can resupply substrate for both PLC γ 2 and class I PI3K by producing PtdIns(4,5)P₂. PIP5K

was shown to enhance the ability of Btk to promote IP₃ production and Ca²⁺ flux following BCR engagement. These and other observations suggest a modification of the signalosome model (Fig. 5B), with the interaction of the PH domain of Btk with PI3K products being important not for activation of Btk kinase, but for positioning Btk in a membrane location where the associated PIP5K can access its substrate.

PLC γ 2, whose activation is the “output” of the signalosome (Fig. 5A), appears to receive both direct and indirect inputs from 3-phosphoinositides. As mentioned, PtdIns(3,4,5)P₃ enhances PLC γ 2 enzyme activity *in vitro* [95], an effect shown to require the SH2 domains of PLC γ 2 isozymes *in vitro* [96]. PtdIns(3,4,5)P₃ indirectly promotes PLC γ 2 activation by binding of domains within PLC γ 2 to help anchor the protein in the same membrane location with Btk and its associated PIP5K. Whether the SH2 domain or the PH domain of PLC γ 2, or both, are important for membrane recruitment of the enzyme has been debated [97,98]. In any event, maximal PLC γ 2 activation is clearly PI3K-dependent, as BCR-stimulated IP₃ production is impaired in p110 δ -deficient B cells [23]. In the absence of PI3K activation, PLC γ 2 might be in a protein complex with Btk/PIP5K via BLNK, and become phosphorylated by Btk and Syk, but the complex would not be properly membrane-targeted.

The establishment of PI3K as a facilitator of signalosome assembly leading to IP₃ generation leads naturally to the assumption that impaired Ca²⁺ signaling is to blame for defective proliferation in PI3K-deficient B cells. However, accumulating evidence suggests that the diminished production of DAG, the other second messenger produced by the action of PLC γ 2, is at least as important for the phenotype. Addition of low concentrations of the DAG analog PdBu, but not calcium ionophore, restores BCR-mediated proliferation in B cells lacking Btk [99]. Conventional isoforms of protein kinase C (PKC) are dependent on both DAG and Ca²⁺ for full activity and are therefore likely to be especially sensitive to graded reductions in PLC γ 2 activity. A critical role for PKC downstream of the signalosome is supported by the observation that PKC inhibitors block proliferation [100]. Moreover, B cells lacking the PKC β isoform fail to proliferate in response to BCR engagement [101].

Signalosome-mediated PKC activation appears essential for activation of the NF κ B pathway. PKC β is required for the activation of I κ B kinase- α (IKK α) following BCR engagement [104,105]. Activation of IKK α leads to the phosphorylation and subsequent degradation of I κ B, which frees the NF κ B transcription factor from sequestration in the cytosol and allows it to translocate to nucleus (Fig. 6). B cells lacking expression of PKC β , p85 α , Btk or BCAP all show impaired NF κ B activation and fail to upregulate the anti-apoptotic NF κ B target gene *Bcl-x_L* [92,102–105]. Importantly, both proliferation and development of p85 α - and Btk-deficient B cells is restored by overexpression of *Bcl-x_L*, and overexpression of c-Rel (a NF κ B isoform) restored function in

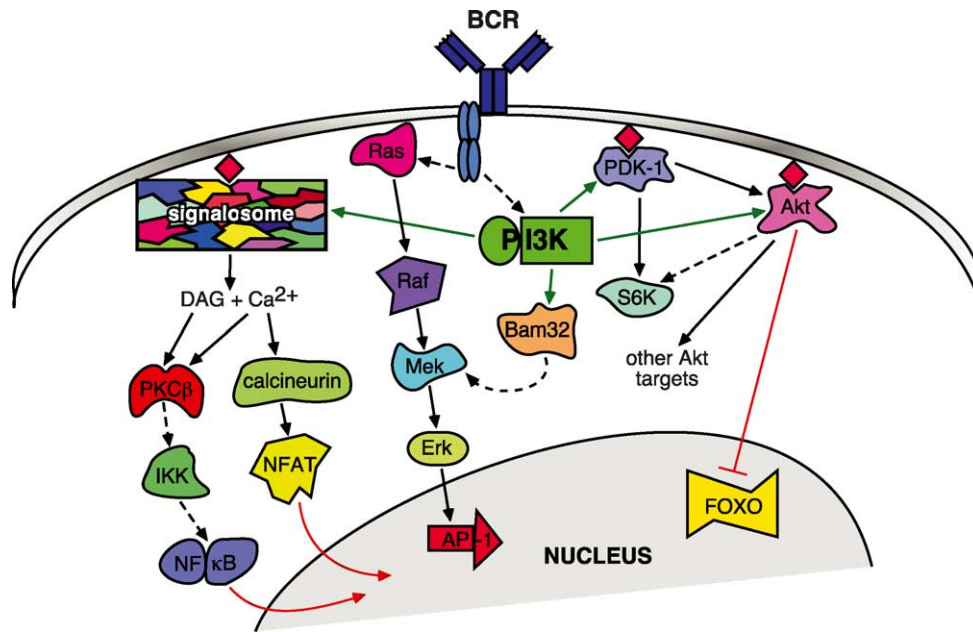


Fig. 6. The role of PI3K in transcription factor regulation in B cells. PI3K-mediated signalosome assembly and the subsequent mobilization of Ca^{2+} and production of DAG lead to the activation and translocation of NF κ B (and probably NFAT) into the nucleus. Activation of Akt downstream of PI3K results in the phosphorylation and inhibition of FOXO family transcription factors, and may also promote NF κ B activation. PI3K might also play a role in the activation of AP-1, as the novel PI3K effector Bam32 contributes to activation of the MAPK cascade. Hatched arrows indicate one or more steps omitted for simplicity, or unknown intermediates. Green arrows represent 3-phosphoinositide-mediated membrane recruitment. Red lines indicate translocation into the nucleus.

BCAP-deficient cells [106]. These findings support a model in which diminished production of DAG and Ca^{2+} in cells lacking signalosome components results in the inability to activate PKC or the NF κ B pathway, and inability to upregulate critical anti-apoptotic target genes (Fig. 6).

It is becoming evident that PI3K activation initiates signals in B cells that are independent of the signalosome linking the BCR to PLC γ 2 activation. In a microarray study of global gene expression in B cells stimulated via the BCR, the number of genes regulated by PI3K alone exceeded the number regulated by both PI3K and Btk [107]. In addition, mice lacking both p85 α and Btk were found to exhibit more severe defects in B cell development than single knockouts [92]. Akt is a critical PI3K effector that transmits proliferation and survival signals in many cell types, and Akt is likely to play a similar role in B cells. Akt becomes phosphorylated and activated following BCR crosslinking, as well as in cells stimulated via TLR4 or CD40 [70,108–110]. Co-ligation of the FC γ RIIB1 inhibitory receptor inhibits BCR-mediated Akt activation [111]. Furthermore, Akt phosphorylation is completely blocked by PI3K inhibitors and partially by loss of p85 α , p110 δ or CD19 [22,23,70,92]. Interestingly, PdBu restores proliferation in p85 α -deficient B cells but not in wild-type cells treated with PI3K inhibitors (D.A.F., unpublished observations). This finding supports the idea that different PI3K effectors are sensitive to graded doses of PI3K signaling. Signalosome assembly and PKC activation appear to be most sensitive and consequently are blocked in cells lacking individual PI3K isoforms, whereas

critical Akt-dependent signals may be partially maintained unless class IA PI3K activity is fully abolished. This model can be tested by generating mice whose B cells lack both p85 α gene products and p85 β . Although deletion of p85 β has no apparent consequences for B cell development, activation and function [112], this isoform may have a required role in the absence of p85 α .

Akt phosphorylates many protein substrates to promote proliferation and survival. Among the ever-growing list of known Akt substrates, those important for B cell activation are not fully established. FOXO transcription factors are a group of Akt substrates known to promote cell cycle arrest, and/or apoptosis in many cell types. These functions are opposed by PI3K/Akt signaling (reviewed in [113,114]). In both primary B cells and B cell tumor lines, BCR engagement is followed by FOXO protein phosphorylation on consensus Akt sites, and subsequent export of FOXO from the nucleus (Fig. 6) [115,116]. Consistent with functional inactivation of FOXO proteins, a number of putative FOXO target genes are downregulated following BCR stimulation in a PI3K-dependent manner [107,116]. Retroviral overexpression of a PI3K-independent version of FOXO in LPS-stimulated primary B cells leads to delayed cell cycle progression and increased levels of death [116]. These findings suggest that FOXO inactivation contributes to PI3K-dependent B cell proliferation and survival.

In T cells, Akt contributes to the activation of the NF κ B pathway and Bcl-x_L upregulation via phosphorylation of Cot/Tpl-2 [117]. A role for PI3K/Akt signaling to NF κ B in

B cells, independent of the Btk/PKC-dependent NF κ B input, has been suggested [92]. It is also likely that Akt mediates the ability of PI3K to promote NF κ B activation in B cells stimulated via TLR4 or CD40 [108,110] two receptors that potently activate NF κ B without inducing Ca²⁺ flux. Another prominent Akt substrate, glycogen synthase kinase-3 (GSK-3), is also phosphorylated and inactivated following BCR engagement [118]. More recent data indicate that GSK-3 phosphorylation in B cells is mediated by PKC rather than Akt [115].

Activation of Akt and several other cellular kinases depends on phosphorylation of a threonine residue in the activation loop by PDK-1 (reviewed in [119]). Although PDK-1 can phosphorylate some substrates in resting cells, production of PtdIns(3,4,5)P₃ is a critical switch that allows PDK-1 to gain access to new substrates. One substrate of PDK-1 that is phosphorylated in a PI3K-dependent manner is S6 kinase (S6K) [120]. This enzyme plays an evolutionarily conserved role in the regulation of protein translation and cell size. S6K activation also requires the activity of the target of rapamycin (TOR). S6K1 is rapidly phosphorylated in splenic B cells stimulated with anti-IgM or LPS in a manner blocked by PI3K inhibitors or rapamycin [121,122]. It is likely that the anti-proliferative effects of these inhibitors in B cells are due in part to blockade of the S6K pathway.

Bam32 is a novel PI3K effector whose PH domain is selective for PtdIns(3,4)P₂ (Fig. 3). Recent studies of Bam32-deficient mouse B cells and DT40 cells suggest that this adapter protein links BCR engagement to activation of the MAP kinase pathways (Fig. 6) [123,124]. This finding may help explain the observation that PI3K inhibitors or loss of p110 δ partially impairs Erk activation in B cells. However, another group reported normal MAP kinase activation in Bam32-deficient B cells [125].

PI3K signaling is generally studied in the context of events that occur seconds or minutes following mitogenic stimulation. However, it is important to keep in mind that PI3K can also have required functions during cell cycle progression, hours after initial activation [126]. We recently demonstrated that treatment of wild-type B cells with PI3K inhibitors as late as 40 h after stimulation blocks cell division and leads to increased apoptosis [122]. The effect was most pronounced in cells stimulated through the BCR but was also observed in cells treated with LPS. In all conditions, the ability to sustain proliferation correlated with activation of S6K and increases in cell size. Of note, c-Myc is a NF κ B target gene in B cells that has been implicated in PI3K-dependent cell size increases following BCR stimulation [127].

6. Conclusions and future directions

Different receptors on B cells set in motion distinct arrays of signal transduction pathways to trigger proliferation and/or differentiation. A striking common feature in many of these pathways is the activation of class I PI3K. In many

cases, PI3K products promote membrane recruitment of critical components of a given pathway, nucleating what has become known as a “signalosome”. In this way, enzymes are brought near their substrates, the scaffolding and signal amplification function of adapter proteins is optimized, and critical enzymes achieve full activation. It appears that PI3K is involved in the construction of multiple signalosomes, the particular composition and specific downstream targets of which are determined by the receptor from which the signal originates. One example is the initiation of both PDK-1/Akt signaling and the Ca²⁺ signaling cascade by antigen receptor engagement, as compared to the exclusive activation of PDK-1/Akt without Ca²⁺ mobilization in B cells stimulated with LPS.

Despite the fact that loss of PI3K function has dramatic consequences to B cell function, there is evidence that a limited set of downstream effectors is critical for signal propagation. In B cells treated with PI3K inhibitors at a concentration that reduces proliferation by 80%, only a small fraction (<5%) of early gene expression changes are significantly altered [107]. Many of these are downregulated genes whose transcription in resting cells may be dependent on FOXO function. Among upregulated genes, Bcl-x_L appears to be a cornerstone of PI3K-dependent B cell activation, as transgenic expression of Bcl-x_L restores development and proliferation in mice lacking p85 α [92]. Although Bcl-x_L is a known target gene for NF κ B, it is worth noting that Bcl-x_L transcription is also repressed by FOXO proteins [128].

Phosphorylation of Akt is often used as a surrogate read-out of PI3K activation. In B cell signaling studies, the impact of genetic or pharmacological PI3K manipulation has often been estimated by changes in Akt phosphorylation. However, it is conceivable that different pools of PI3K and its products regulate Akt and other effectors. In addition, preparations of primary B cells from mice or humans are heterogeneous mixtures of cells representing different developmental stages with distinct activation properties. Thus, measurement of 3-phosphoinositide concentrations in cell subsets and at subcellular resolution provides a more accurate and informative picture. Procedures to measure PtdIns(3,4,5)P₃ in single cells by FACS [26,129], or confocal imaging of cells expressing PH domain-GFP fusions [16,17,130], are beginning to be applied to this important question. It will be important to use these emerging technologies to quantitate and localize PI3K signaling in different B cell subsets following treatment with different mitogens that activate class IA PI3K, as well as in cells treated with chemokines and other GPCR ligands that activate class IB PI3K. Single cell technologies can also be applied to the study of which PI3K effectors and target genes are involved in responses to different stimuli.

Acknowledgements

Unpublished data from our lab that is cited in this review was supported by NIH grant AI-50831.

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