

PHOSPHOINOSITIDE 3-KINASE: Diverse Roles in Immune Cell Activation

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■ **Abstract** Cells of the immune system carry out diverse functions that are controlled by surface receptors for antigen, costimulatory molecules, cytokines, chemokines, and other ligands. A shared feature of signal transduction downstream of most receptors on immune cells, as in nonhematopoietic cell types, is the activation of phosphoinositide 3-kinase (PI3K). The mechanism by which this common signaling event is elicited by distinct receptors and contributes to unique functional outcomes is an intriguing puzzle. Understanding how specificity is achieved in PI3K signaling is of particular significance because altered regulation of this pathway is observed in many disease states, including leukemia and lymphoma. Here we review recent advances in the understanding of PI3K signaling mechanisms in different immune cells and receptor systems. We emphasize the concept that PI3K and its products are components of complex networks of interacting proteins and second messengers, rather than simple links in linear signaling cascades.

INTRODUCTION

The term phosphoinositide 3-kinase (PI3K; several other names appear in the literature, including PI 3-kinase and phosphatidylinositol 3-kinase) refers to a family of enzymes that phosphorylate D-*myo*-phosphatidylinositol (PtdIns) or its derivatives on the 3-hydroxyl of the inositol head group (Figure 1). The primary function of 3-phosphorylated inositol lipids (3-phosphoinositides) is to serve as membrane targeting signals to mediate membrane recruitment of selected proteins. PI3Ks and their function in regulated protein translocation are conserved through eukaryotes. In nearly all cases studied, genetic ablation or enhancement of PI3K function has a profound effect on cellular and organismal function (1-4).

Most early studies of PI3K essentially catalogued the receptors that activate the enzyme and the cellular responses that ensue. Experimental approaches employed in this work included the use of pharmacological inhibitors wortmannin and LY294002, expression of mutated forms of PI3K genes and/or upstream receptors,

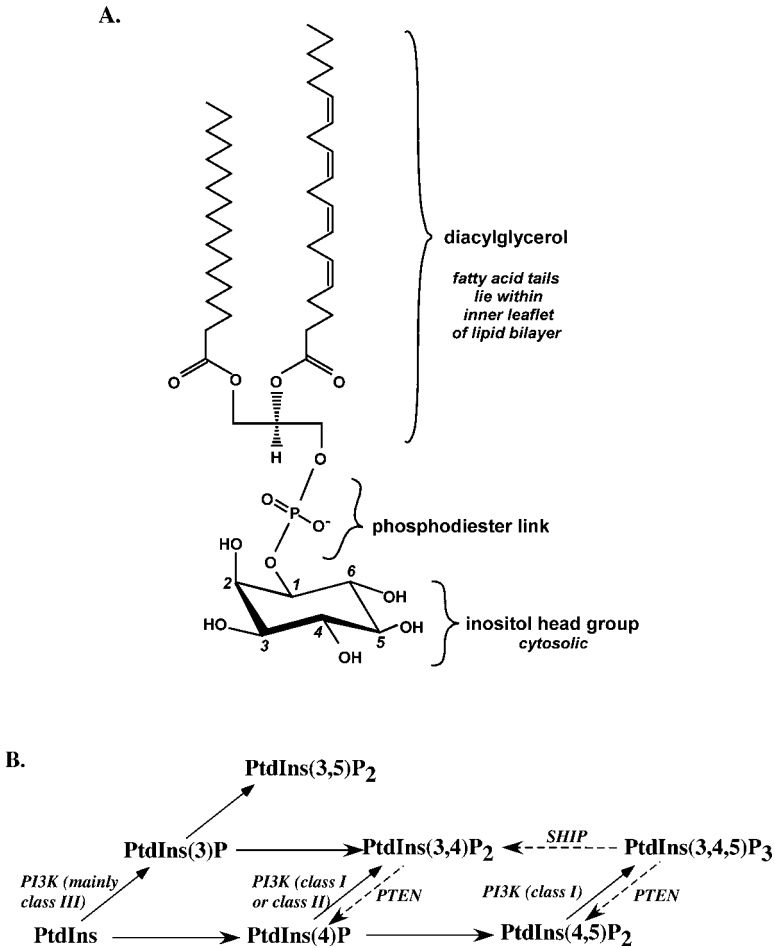


Figure 1 Structure and synthesis of 3-phosphoinositides. (A) Chemical structure of D-myo-phosphatidylinositol (PtdIns). With permission, from the *Annual Review of Biochemistry*, Volume 70 ©2001 by Annual Reviews, www.AnnualReviews.org. (B) Diagram of pathways for synthesis and degradation of D-3-phosphoinositides. Enzymes involved in this pathway that are not discussed in the text are not included.

and measurement of cellular 3-phosphoinositide levels by chromatographic methods. Together these approaches made clear that elevation in 3-phosphoinositides is characteristic of a host of responses to a great variety of stimuli, including those important for immune responses. A subsequent phase of PI3K research involved the identification of cellular proteins that bind selectively to 3-phosphoinositides. The list of modular lipid binding domains and the proteins in which they are found, herein termed PI3K effectors, is ever growing.

Although the cataloguing of PI3K signaling responses and effectors continues, progress in recent years has been driven by methodological innovations that have allowed a greater mechanistic understanding of this important signaling pathway in different cell types. Knockout and knock-in mouse models have helped define the roles of individual PI3K genes in normal physiology, overcoming the limitations of global inhibitors and dominant negatives. The development of fluorescent probes to localize and quantitate 3-phosphoinositides has enabled the comparison of lipid distribution within and between cells that could not be achieved by earlier biochemical methods. Expanded studies of PI3K signaling in primary cells have helped to resolve controversies arising from the study of cultured cell lines.

This review presents a brief overview of PI3K structure, activation mechanism, and function (for more extensive reviews, see 1, 5). We then describe notable recent advances in the understanding of PI3K signaling in cells of the immune system, with emphasis on results gained from primary cells studied with the novel approaches outlined above. Headings are organized according to types of receptor signaling systems rather than by cell types. We focus on physiological responses of mature cells; the reader is referred to detailed reviews on the role of PI3K in lymphocyte development, leukemogenesis, and autoimmunity (4, 6, 7). Space constraints limit discussion of PI3K function downstream of receptors for mitogenic cytokines and inflammatory mediators (for recent reviews, see 2, 8).

PI3K BACKGROUND

Phosphoinositides and PI3K Enzyme Families

Phosphoinositides are found in the cytoplasmic leaflet of cellular membranes where they regulate activities that include vesicle trafficking, cytoskeletal reorganization, and signal transduction (1, 9). Among the 3-phosphoinositides, PtdIns(3)P is the most abundant and its levels are relatively constant; it is primarily found in endosomes where its major function appears to be in protein sorting, though acute increases in PtdIns(3)P synthesis play a role in specific processes such as phagocytosis (10). Little is known about the regulation or function of PtdIns(3,5)P₂ in mammalian cells. PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ (also known as PIP₃) are essentially absent in quiescent cells but are transiently increased at the plasma membrane in response to a broad array of extracellular cues. Although these lipids contribute to a host of cellular responses, their production is strongly correlated with proliferation and survival in many cell types (1, 2, 7).

PI3Ks are categorized as class I, II, or III, depending on their subunit structure, regulation, and substrate selectivity (Figure 2) (1, 5). Class I PI3Ks are the only enzymes capable of converting PtdIns(4,5)P₂ to the critical second messenger PtdIns(3,4,5)P₃. Class I PI3Ks are heterodimers composed of a catalytic subunit of approximately 110 kDa, and a tightly associated regulatory subunit that modulates its activity and cellular location. The class IA subgroup exists as multiple isoforms, with three catalytic subunits (p110 α , p110 β , and p110 δ) encoded by three distinct

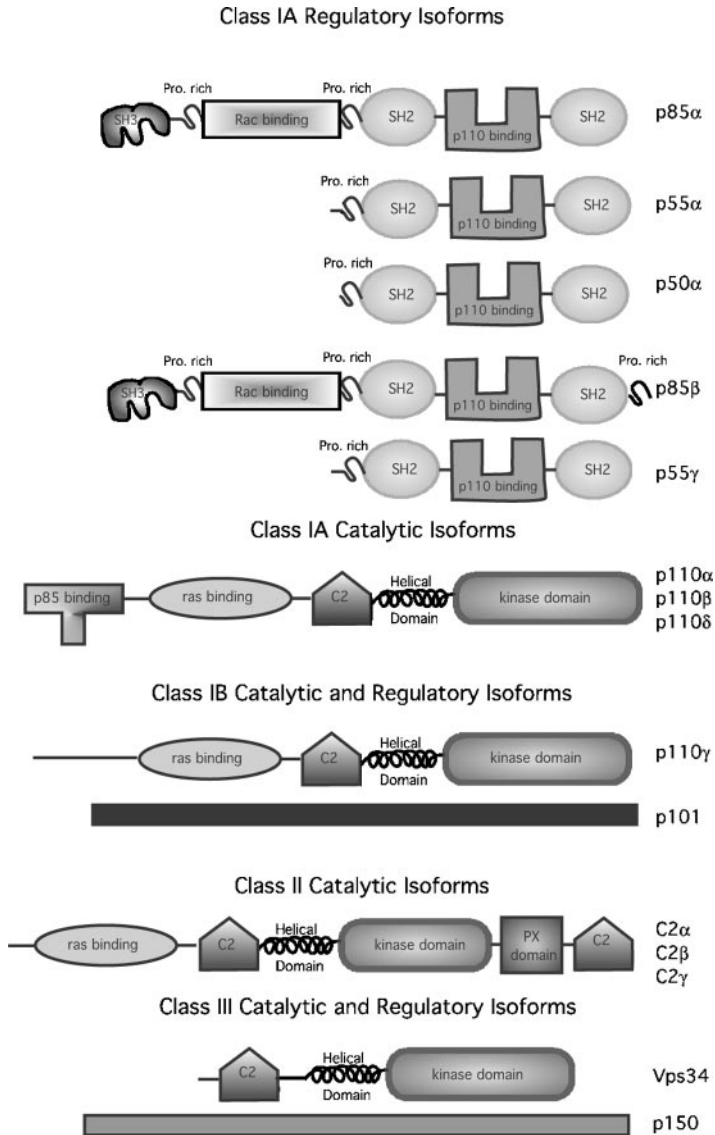


Figure 2 Schematic diagram of the PI3K catalytic and regulatory subunits. Class IA regulatory subunits have several modular domains that can regulate function of the heterodimer. Each isoform has two SH2 domains selective for binding pTyr-X-X-Met sequences, an interaction that appears critical for enzyme activation. The Rac-binding domain in p85 α and p85 β , also known as the breakpoint cluster region homology or BH domain, is homologous to RacGAPs for Rho family small G proteins but lacks GAP activity. The SH3 and proline-rich motifs can also participate in intramolecular and intermolecular interactions.

genes and five regulatory subunits (p85 α , p85 β , p55 γ , p55 α , and p50 α) encoded by three genes (Figure 2). Alternative transcripts of the *Pik3r1* gene encode the p85 α , p55 α , and p50 α proteins. A single class IB isoform is composed of the p110 γ catalytic subunit and the p101 regulatory subunit (Figure 2). Class IB PI3K is expressed most highly in cells of the immune system (1). Most of the immune receptors discussed in this review activate class IA and/or IB PI3Ks, leading to the production of PtdIns(3,4,5)P₃.

The class II PI3K subgroup in mammals comprises three catalytic isoforms (α , β , and γ) that are larger in size than those of class I PI3Ks and do not constitutively associate with a regulatory subunit (Figure 2) (1, 5). These enzymes selectively use PtdIns as a substrate to produce PtdIns(3)P, although they may also contribute to the production of PtdIns(3,4)P₂. Although some data suggest that class II PI3K enzyme activity and location are regulated by extracellular signals, little is known about their function downstream of immune cell receptors. A single isoform of class III PI3K is found in organisms ranging from yeast to humans. Class III PI3K is only able to produce PtdIns(3)P, and is localized to endocytic vesicles where its major function appears to be in protein and vesicle trafficking. There is growing evidence that class III PI3K contributes to trafficking processes unique to immune cells, especially phagocytosis (10).

Activation and Regulation of Class I PI3Ks

Class I PI3Ks in resting cells are cytoplasmic proteins whose substrates reside in cellular membranes (1, 5). A number of protein-protein interactions contribute to the proper localization and activation of these enzymes following cell stimulation. For class IA PI3Ks, the best-understood mechanism of activation involves the binding of two Src-Homology 2 (SH2) domains found in all forms of the regulatory subunit (Figure 2) to phosphorylated tyrosines (pTyr) within the sequence context pTyr-X-X-Met (X is any amino acid). This SH2-pTyr interaction is triggered by receptors with either intrinsic or associated tyrosine kinase activity, bringing PI3K to membrane-associated signaling complexes and, in many cases, allowing further activation by additional interactions. Of particular importance for PI3K function are small G proteins of the Ras and Rho families. GTP-bound Ras binds to class IA catalytic subunits, an interaction shown recently to activate PI3K only in the context of a SH2-pTyr interaction (11). Rac proteins represent a subgroup of Rho family G proteins that when bound to GTP can stimulate PI3K activity by two potential mechanisms. Rac-GTP can bind directly to a domain in p85 α and/or p85 β that shows homology to GTPase-activating proteins (GAPs) for Rho family members (Figure 2). In addition, Rac-GTP associates with PtdIns(4)P-5-kinase (PIP5K), an enzyme that can generate local increases in PtdIns(4,5)P₂, the substrate for PI3K (12). Additional modular domains within the amino-terminal portions of p85 α and p85 β can mediate further associations that regulate PI3K function (see legend to Figure 2).

Whereas the class IA PI3Ks are activated primarily by signaling pathways that involve tyrosine kinase activation, the class IB PI3K is activated by $\beta\gamma$ subunits

of heterotrimeric G proteins (1, 5). A growing list of G protein-coupled receptors (GPCRs) have been demonstrated to trigger PI3K pathway activation, and genetic evidence exists for a required function of class IB PI3K in PtdIns(3,4,5)P₃ production triggered by certain GPCRs on leukocytes (13–15). The overall structure of the p110 γ catalytic isoform is similar to the class IA enzymes, including a Ras-binding domain (Figure 2). However, the amino terminus of p110 γ is distinct and mediates interaction with the p101 subunit, whose structure does not contain recognizable sequence motifs yet appears to be required for class IB enzyme activation by $\beta\gamma$ subunits.

Multiple PI3K Effectors

A number of modular protein domains have evolved to recognize specific phosphoinositides (1, 16–18). The pleckstrin homology (PH) domain is a small (~60 aa) module found in more than 100 proteins. Although most PH domains have demonstrable affinity for phosphoinositides, only a subset is selective for PI3K products. Among this group, further specificity has been defined, with some members selective for PtdIns(3,4,5)P₃ or PtdIns(3,4)P₂ and others capable of binding either lipid with equivalent affinity (17). The phox homology (PX) domain has a distinct structure from PH domains but the family also contains members that bind selectively to one or more 3-phosphoinositides (18). The FYVE domain (originally named based on the first letter of four yeast proteins with the module) was identified as a PtdIns(3)P-specific binding module and is found in a number of proteins involved in protein and vesicle trafficking (16).

Several proteins have been identified as likely “PI3K effectors” based on the presence of a domain selective for one or more 3-phosphoinositides (1, 2). In general, proteins with PH and PX domains are involved in signal transduction, whereas proteins with FYVE domains regulate trafficking. Phosphoinositide binding by a given PH or PX domain in some cases can directly increase activity of linked enzymatic domains, by allosteric changes and/or relieving intramolecular inhibitory interactions. However, in most cases, the membrane recruitment of a PI3K effector is one step in an activation process that can also involve posttranslational modification (i.e., phosphorylation) and additional intermolecular associations. Indeed, a paradigm has emerged in antigen receptor signaling in which PI3K lipid production is one of several crucial steps leading to the formation of large signaling complexes, or “signalosomes,” composed of numerous enzymes and scaffolding/adaptor proteins (19, 20).

Two groups of PI3K effectors that have received particular attention in studies of immune cell signaling are tyrosine kinases of the Tec family and serine/threonine kinases of the AGC family (21, 22). Members of the Tec family include Tec itself, Btk, Itk, Etk, and Rlk. Each member of the Tec family, except Rlk, possesses a PH domain with apparent selectivity for PtdIns(3,4,5)P₃. Traditional models have posited that PtdIns(3,4,5)P₃ binding by Tec family PH domains is critical for activating tyrosine kinase activity, in part by facilitating activation loop

phosphorylation by membrane-associated Src family kinases (20, 21). However, recent data have challenged that view, and modifications to this model are described below. AGC family serine/threonine kinases regulated by PI3K include phosphoinositide-dependent kinase (PDK-1), Akt (also termed PKB), certain isoforms of protein kinase C (PKC), and S6 kinase. Akt exists as three isoforms and its activation is strongly linked to PI3K-dependent proliferation and survival signals. Akt activation depends upon phosphorylation by PDK-1, which also has a 3-phosphoinositide-selective PH domain that brings it to the membrane to interact with Akt. Other important PDK-1 substrates include PKC and S6 kinase.

Of note, PH and FYVE domains linked to green fluorescent protein (GFP) or its derivatives have proven highly useful as genetically encoded fusion protein probes for localization of specific 3-phosphoinositides by fluorescence microscopy. Many of the papers cited herein utilize fusions of fluorescent proteins with the PH domain of Akt to detect PtdIns(3,4,5)P₃, and/or fusions with tandem FYVE domains from the protein EEA1 to detect PtdIns(3)P.

Phosphatases

Two enzymes are primarily responsible for dephosphorylating PtdIns(3,4,5)P₃: PTEN (Phosphatase and TENsin homolog) and SHIP (SH2-containing inositol phosphatase) (1). PTEN hydrolyzes the 3-phosphate and plays a central role in limiting cellular levels of PtdIns(3,4,5)P₃, thereby opposing proliferation and survival responses (23). Loss of PTEN function is observed in a large fraction of human cancers (7). Much has been learned in recent years about PTEN function in immune cells (24). SHIP1 and SHIP2 are SH2 domain-containing phosphoinositide phosphatases that selectively remove the 5-phosphate from PtdIns(3,4,5)P₃ to generate PtdIns(3,4)P₂. Thus, SHIP1/2 activity may alter the spectrum of PI3K-dependent signals rather than simply opposing all PI3K signaling. SHIP1 is selectively expressed in cells of the immune system and is important for setting activation thresholds and maintaining homeostasis of a variety of hematopoietic lineages (25).

PI3K IN ANTIGEN RECEPTOR AND Fc RECEPTOR SIGNALING

Signal transduction pathways initiated by the B cell receptor (BCR), T cell receptor (TCR), high-affinity IgE receptor (FcεRI), and Fcγ receptors share many common features (19, 26–28). Clustering of the receptors triggers the activation of Src family tyrosine kinases that phosphorylate ITAMs (immunoreceptor tyrosine-based activation motifs) in the cytoplasmic tails of receptor signaling chains. Subsequent activation of Syk/ZAP-70 family and Tec family tyrosine kinases controls downstream events including generation of sustained calcium (Ca²⁺) mobilization, GTP loading of small G proteins, and initiation of mitogen-activated protein

(MAP) kinase cascades. Each receptor also triggers the accumulation of PI3K products and activation of Akt. Many antigen-dependent functional responses of primary cells are blocked by PI3K inhibitors (2). However, important differences exist among antigen and Fc receptor systems in the mechanisms by which PI3K and its effectors are activated, and the specific PI3K isoforms involved.

B Cell Receptor

The mechanisms by which BCR engagement leads to the recruitment and activation of PI3K are complex. Many clues have been provided by studies in the DT40 B lymphoma system, a chicken cell line in which genes can be readily disrupted by homologous recombination. DT40 cells lacking Syk exhibit a severe reduction in BCR-dependent PI3K activation, as determined by PtdIns(3,4,5)P₃ measurements (29). Several proteins in B cells are likely targets for Syk-mediated phosphorylation of Tyr-X-X-Met motifs, including CD19 and the adapter proteins BCAP and Gab1.

CD19 is a major contributor to PI3K activation in mouse B cells (3, 4). This transmembrane protein is loosely associated with the BCR and the cytoplasmic tail of CD19 contains tandem pTyr-X-X-Met motifs that become phosphorylated following BCR crosslinking. Co-crosslinking CD19 with the BCR augments PI3K activation and increases the sensitivity of B cell responses by 3 to 4 orders of magnitude. Transgenic and knockout studies have confirmed that CD19 plays a role both in B cell development and in setting the activation threshold for mature B cells (30). In splenic B cells from CD19 knockout mice, anti-Ig-stimulated Akt activation is reduced by greater than tenfold (31). Two recent studies showed elegantly that activation of PI3K is the primary signaling function of CD19. In the first report, a CD19 transgene with mutations in the tyrosine residues that interact with PI3K was unable to restore function when introduced into CD19-deficient mice (32). The second study showed that the developmental and functional phenotypes of CD19 knockout mice could be complemented by B cell-specific deletion of the PTEN phosphatase (33). This paper also used flow cytometry (FACS) to measure PI3K activation with anti-PtdIns(3,4,5)P₃ antibodies, a novel assay that should facilitate quantitative measurements of PI3K signaling in lymphocyte subsets and when cell yield is limiting (see also 34, 35).

The adapter protein BCAP has four Tyr-X-X-Met motifs and BCAP-deficient DT40 cells have greatly impaired activation of PI3K and Akt following engagement of either the BCR or CD19 (36). However, targeting of the mouse BCAP gene had no effect on activation of PI3K or Akt in splenic B cells stimulated with anti-IgM (37). BCAP-deficient B cells did show impaired Ca²⁺ mobilization and proliferation. Gab1 is a member of a subgroup of adapter proteins (also including Gab2 and Gab3) that can serve as response amplifiers in PI3K signaling (38). These proteins possess PH domains selective for PtdIns(3,4,5)P₃ and are recruited to membranes following PI3K activation. Subsequent phosphorylation on specific pTyr residues recruits more PI3K. Overexpression of Gab1 in an immature B cell line suggested that Gab1 can augment BCR-mediated Akt activation (39).

However, analysis of Gab1-deficient mouse B cells revealed an inhibitory role for Gab1 in antibody responses to T-independent type-2 antigens (40). Other studies are consistent with the notion that Gab family adapter proteins may have both positive and negative influences on cell activation (41).

Evidence is emerging that Vav proteins also contribute to PI3K activation downstream of the BCR and related receptors. Vav1, Vav2, and Vav3 have guanine nucleotide exchange factor (GEF) activity for Rac family small G proteins, and GTP-bound Rac proteins can increase 3-phosphoinositide production by two mechanisms, as described above. Thus, Vav proteins might contribute to PI3K activation by increasing the fraction of Rac-GTP. Consistent with this, PI3K activation is reduced in DT40 cells lacking Vav3 or expressing dominant negative Rac1 (42). Mouse B cells express both Vav1 and Vav2 and combined deletion impairs B cell development and function to a degree similar to loss of specific PI3K gene products (43, 44). Vav proteins have traditionally been considered to act downstream of PI3K, as they possess PH domains and GEF activity of Vav1 was reported to be enhanced by PtdIns(3,4,5)P₃ (45). However, it has not been conclusively demonstrated that PI3K activation is required for Vav activation in cells (1). To summarize the available data from BCR signaling studies, PI3K activation is driven by interactions with phosphorylated CD19 and probably Rac-GTP, whereas the functions of BCAP and Gab1 adapter proteins with respect to PI3K are not yet clear.

Of the multiple catalytic and regulatory isoforms of class IA PI3K expressed in B cells, unique roles have been identified for p85 α and p110 δ . Two strains of p85 α -deficient mice have been generated. One lacks expression of all gene products including p85 α , p55 α , and p50 α , whereas the other lacks only p85 α . Both strains exhibit defects in development, proliferation, and survival (46, 47). Mice with a null mutation in the p110 δ gene, or with a kinase-dead knock-in mutation, exhibit defects in B cell development, activation, and antibody response that are generally similar to those in p85 α -deficient mice (48–50). B cell function is apparently normal in mice lacking p85 β (J. Deane, M. Trifilo, C. Yballe, S. Choi, T. Lane, D. Fruman, submitted manuscript).

Proliferation in anti-Ig-stimulated B cells lacking p85 α or p110 δ is reduced to a degree similar to that in wild-type cells treated with global PI3K inhibitors, suggesting that p85 α /p110 δ complexes are responsible for a major fraction of PI3K signaling output. This idea was supported by one study of p110 δ -deficient B cells, in which PtdIns(3,4,5)P₃ generation was found to be completely blocked (49). Consistent with failure to generate PtdIns(3,4,5)P₃, Akt phosphorylation was nearly abolished (48–50), as in wild-type cells treated with PI3K inhibitors. Although PtdIns(3,4,5)P₃ measurements have not been reported in p85 α -deficient B cells, one group demonstrated markedly reduced Akt activation (52). Ca²⁺ mobilization in response to anti-Ig, shown previously to be attenuated in primary B cells treated with wortmannin (53), is moderately to severely impaired in cells deficient in p85 α (our unpublished data) or p110 δ (48–50). Of note, Ca²⁺ mobilization and Akt activation are also impaired by coengagement of the BCR with the inhibitory

receptor $Fc\gamma RIIB1$, which recruits SHIP, leading to metabolism of PI3K lipid products (2).

Similar defects in B cell development, function, and Ca^{2+} mobilization are observed in mice lacking either $p85\alpha$, $p110\delta$, Btk, Vav1/Vav2, CD19, BCAP, the adapter BLNK (B cell linker), or phospholipase C- γ -2 (PLC γ 2) (2, 20, 37, 43, 44, 46–50). Ca^{2+} mobilization is also impaired in Rac2-deficient B cells (54). Although detailed comparison of these different genetic models reveals important differences, especially with respect to development and B cell subset differentiation (4), the overall similarity in phenotypes has suggested that these gene products function in a common pathway leading to Ca^{2+} mobilization (20). The intricate signaling connections among these components has suggested a “signalosome” model in which BCR engagement leads to assembly of a large complex of signaling proteins, including both upstream activators (CD19, Vav/Rac, BCAP) and downstream effectors (Btk, PLC γ 2) of PI3K (Figure 3A).

3-Phosphoinositides are thought to help with assembly and proper localization of the signalosome at the B cell membrane. In particular, various observations suggested that binding of the Btk PH domain to $PtdIns(3,4,5)P_3$ was essential for Btk activation and subsequent phosphorylation of PLC γ 2 (20). Especially striking is the finding that *Xid* mice, which harbor a naturally occurring point mutation in the Btk PH domain that impairs binding to $PtdIns(3,4,5)P_3$, have B cell defects nearly as severe as those in Btk-null mice (55). Consistent with these findings, one group reported that $p110\delta$ -deficient B cells exhibit impaired phosphorylation of tyrosines in the Btk activation loop and an autophosphorylation site (49). In contrast, others have reported that Btk kinase activation and PLC γ 2 phosphorylation are intact in B cells lacking either $p85\alpha$ or $p110\delta$, as well as in wild-type cells treated with PI3K inhibitors (50, 52). The basis for the discrepant findings is not fully clear, but may be in part due to the use of different antibodies for detection of Btk phosphorylation (4). However, some of the conflicting data might be reconciled by the recent finding of a new kinase-independent function for Btk: association of the PH domain with $PtdIns(4)P$ -5-kinase (PIP5K) (56). This enzyme can generate $PtdIns(4,5)P_2$, the substrate for PLC γ 2. Thus, a modified model can be proposed in which $PtdIns(3,4,5)P_3$ is not essential for Btk activation or phosphorylation of PLC γ 2, but that proper membrane targeting by $PtdIns(3,4,5)P_3$ allows Btk to bring along an enzyme necessary to generate locally high concentrations of $PtdIns(4,5)P_2$, allowing PLC γ 2 to generate sufficient IP_3 levels for maximal Ca^{2+} mobilization (Figure 3A). This mechanism could help solve the problem of potential competition between PLC γ 2 and PI3K for the same substrate. However, Btk-mediated increases in local $PtdIns(4,5)P_2$ production do not appear to be necessary for resupply of substrate for PI3K, as activation of Akt is intact in Btk-deficient B cells (52).

Activation of PLC γ 2 in the signalosome leads to the production of two second messengers, diacylglycerol (DAG) and IP_3 , the latter important for triggering Ca^{2+} release. Both DAG and Ca^{2+} contribute to the activation of conventional PKC isoforms. The defect in proliferation to anti-Ig in B cells lacking Btk or $p85\alpha$

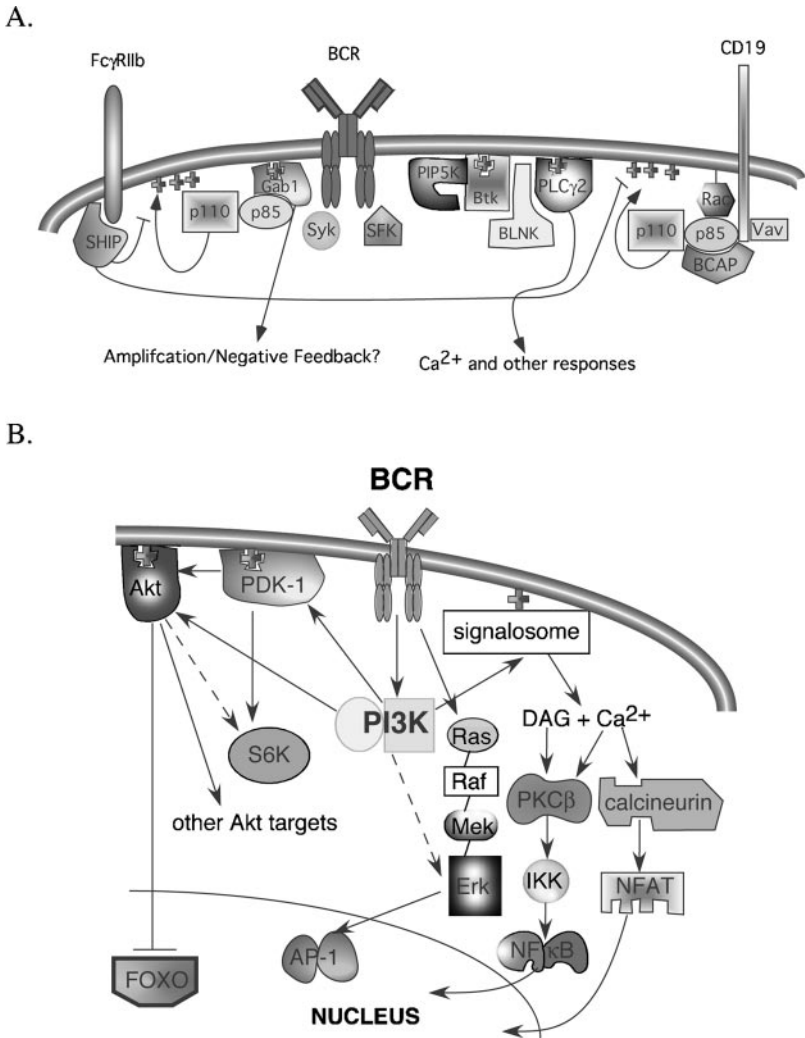


Figure 3 Role of PI3K in early BCR signaling and transcription factor activation. Cross shapes in this figure and Figures 4–7 represent $\text{PtdIns}(3,4,5)\text{P}_3$. (A) Current model for activation of PI3K and its effectors, including components of the BCR signalosome that promotes Ca^{2+} mobilization. $\text{PtdIns}(3,4,5)\text{P}_3$ is important for proper assembly of the complex at the membrane but its role in activation of Btk has been debated (see text). Blockade by the inhibitory receptor $\text{Fc}\gamma\text{RIIB1}$ is also shown. SFK = Src-family kinase. (B) Diagram showing how PI3K can regulate multiple critical transcription factors in B cells. The activity of $\text{NF}\kappa\text{B}$, AP-1, and NFAT can be enhanced, whereas FOXO function is suppressed. Another downstream target shown, S6 kinase (S6K), participates in regulation of mRNA translation. Hatched lines in this figure and others depict pathways with one or more steps omitted for simplicity.

can be restored by low concentrations of phorbol ester, a DAG analog (57; our unpublished data). This suggests that failure to generate DAG and activate PKC is the central signaling defect in these cells. The PKC β isoform may be critical in this pathway as B cells lacking PKC β fail to proliferate to anti-Ig (58).

This link has been strengthened by the finding that B cells lacking Btk, p85 α , BCAP, or PKC β are all defective in activation of the NF κ B pathway and upregulation of the NF κ B target gene *Bcl-x_L* (52, 59–63). NF κ B transcription factors are sequestered in the cytoplasm by I κ B subunits, but are released for nuclear entry following I κ B phosphorylation by the I κ B kinase (IKK) complex. PKC β is required for BCR-mediated activation of IKK α (61, 62). The importance of IKK α and other components of the NF κ B pathway for B cell survival and function is well-established (64 and references therein). Notably, overexpression of a *Bcl-x_L* transgene in B cells is sufficient to restore development and proliferation in B cells lacking Btk or p85 α (52, 65). Failure to upregulate cyclin D2 expression is a common defect in cells lacking signalosome components or treated with PKC inhibitors (52, 66–68); however, this may be a direct consequence of impaired *Bcl-x_L* induction leading to cell death (52).

Several observations suggest that PI3K activation in B cells triggers important signals that are independent of Btk. Mice lacking both p85 α and Btk have more severe B cell defects than single knockouts have (52). In addition, PI3K and Btk have both shared and distinct target genes, as determined by microarray analysis (69). PDK-1 and Akt are likely mediators of critical Btk-independent signals downstream of PI3K. Akt phosphorylates and inactivates FOXO proteins, a family of transcription factors that promote quiescence (70). Several potential FOXO target genes are not appropriately downregulated in PI3K-deficient cells (69, 70). Akt and novel PKC isoforms (also PDK-1 substrates) may also feed into the NF κ B pathway independently of the signalosome and PKC β (52, 71, 72). In addition, PDK-1 and Akt contribute to the activation of the S6 kinase pathway and subsequent increases in translation and cell size that are critical for cell cycle progression (73, 74). PI3K also promotes B cell size increases via the NF κ B-dependent upregulation of c-Myc (75). In this regard, we recently reported that sustained PI3K activation and concomitant increases in cell size are required for cell cycle progression and for continued mitotic activity of daughter cells (76).

Although activation of the NF κ B pathway and inactivation of FOXO proteins have emerged as critical downstream events in PI3K signaling in B cells, PI3K also can influence other key transcription factors (Figure 3B). PI3K activation could promote NFAT nuclear accumulation in two ways: by enhancing Ca²⁺ mobilization and calcineurin-dependent NFAT dephosphorylation, leading to nuclear import, and by Akt-mediated inactivation of GSK-3 (glycogen synthase kinase-3), a kinase that can phosphorylate NFAT and drive nuclear export (72). PI3K inhibitors have been reported to diminish BCR-dependent activation of Erk, likely leading to impaired AP-1 transcriptional activation (77).

Studies of B cells lacking PTEN or SHIP1 have demonstrated that precise regulation of PI3K signaling is essential for normal BCR responses. In PTEN-deficient

B cells, treatment with anti-Ig results in elevated production of PtdIns(3,4,5)P₃, enhanced and sustained phosphorylation of Akt, and markedly increased proliferation (33, 78). However, class switch recombination is impaired, owing to an inability to induce expression of activation-induced cytidine deaminase. As might be expected, one group reported enhanced survival of PTEN-deficient B cells (78); in contrast, another group reported increased susceptibility to apoptosis, which they attributed to inappropriate cell cycle entry (33). Analysis of SHIP1-deficient B cells confirmed its central role in inhibitory signaling downstream of Fc γ RIIB1, and showed that loss of this control mechanism correlated with elevated basal levels of serum Ig (79, 80).

T Cell Receptor

Physiological engagement of the TCR triggers the rapid and sustained production of PtdIns(3,4,5)P₃. Elegant studies of T cells expressing GFP-PH domain fusion probes showed that the lipid is concentrated at the site of antigen contact, although it distributes throughout the plasma membrane (81–83). When PI3K inhibitors or antibodies that block TCR-MHC contact are added at various time points after activation, PtdIns(3,4,5)P₃ disappears rapidly from the membrane, indicating that active phosphatases turn off the signal in the absence of prolonged TCR engagement and PI3K enzyme activation (81, 83). 3-Phosphoinositide production appears functionally important in primary T cells as PI3K inhibitors block proliferation driven by antigen, and in some experiments, anti-CD3 ϵ antibodies. Moreover, studies of T cells lacking PTEN or expressing activated forms of PI3K or Akt have uniformly supported a role for the PI3K/Akt pathway in promoting T cell proliferation and survival (6). On the other hand, some experiments have suggested that PI3K activation plays a role in opposing T cell-activation signals (72, 84). Some of these data were gathered from studies of the Jurkat cell line, which has aberrant PI3K signaling due to the absence of PTEN and SHIP1 (84). Nevertheless, it is becoming clear that the inputs and outputs of PI3K signaling in T cells are distinct in many ways from B cells.

The process of antigen recognition by T cells is more complex than that of B cells. TCR engagement by MHC-peptide on antigen-presenting cells (APC) is accompanied by multiple receptor-ligand interactions that can influence signaling. Indeed, productive T cell activation requires coreceptor engagement (CD4 or CD8) and a second costimulatory signal in addition to the primary TCR-mediated set of signals. Even purified T cells stimulated with crosslinking antibodies may receive signals from neighboring cells. Thus, it has been difficult to study TCR activation of PI3K in isolation. To briefly review data acquired primarily from antibody crosslinking experiments in T cell lines, TCR engagement has been reported to lead to association of p85 regulatory subunits with Rac1 and various adapter proteins, as well as cytoplasmic domains of the TCR signaling chains (72). There are few definitive data establishing a role for these interactions in PI3K activation in primary mature T cells; however, the link between Rac and PI3K was strengthened

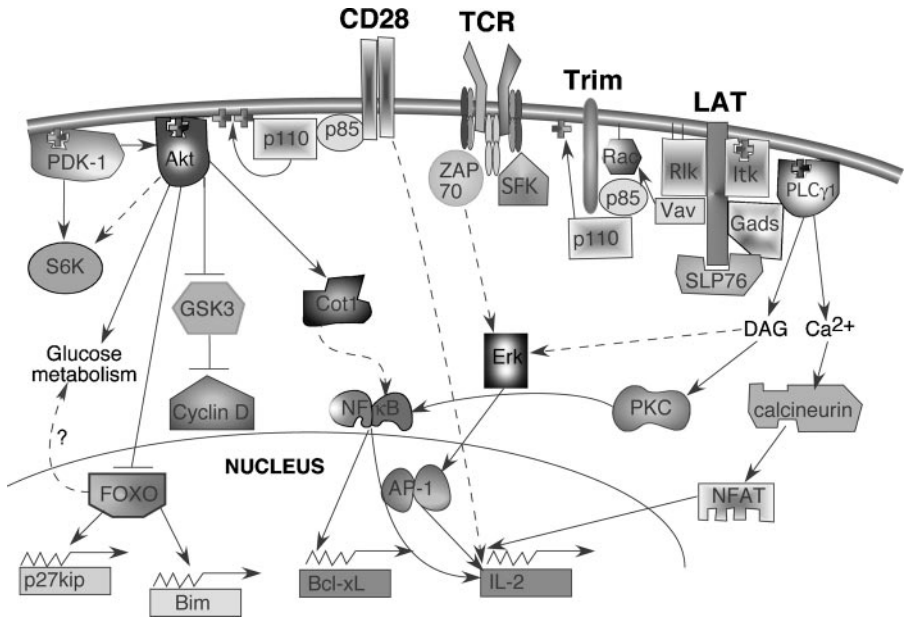


Figure 4 Role of PI3K in TCR signaling and regulation of gene expression. The costimulatory receptor CD28 is shown as the major contributor to PI3K/Akt activation but signaling from the TCR itself can also activate PI3K. 3-Phosphoinositides may not be required for Ca²⁺ flux and Ca²⁺-dependent events in T cells due to the expression of Rlk.

by the finding that levels of Rac-GTP and Akt phosphorylation in TCR-activated thymocytes are markedly reduced in the absence of Vav1 (85) (Figure 4).

One clear mechanism to activate PI3K in T cells is via engagement of costimulatory receptors CD28 or ICOS (inducible costimulator) (Figure 4). CD28 is the primary costimulatory molecule on resting T cells, and ligation of CD28 with antibodies or physiological ligands of the B7 family results in rapid activation of PI3K and Akt. The cytoplasmic tail of CD28 contains a Tyr-Met-Gln-Met sequence that can bind p85 regulatory subunits when phosphorylated. Notably, the Gln residue also makes the phosphotyrosine residue competent to bind the adapter protein Grb2, perhaps in competition with PI3K. The ICOS molecule is related to CD28 but binds distinct ligands and appears to function more in responses of activated T cells than in initial activation. The ICOS cytoplasmic tail contains a sequence Tyr-Met-Phe-Met that can bind PI3K but not Grb2. ICOS engagement leads to remarkably greater recruitment of PI3K than CD28 engagement (86).

Studies of T cell function in mice lacking class IA regulatory isoforms have yielded unexpected results. The predominant regulatory isoform p85 α (or p85 α /p55 α /p50 α), while critical for B cell function, is not essential for proliferation driven by the TCR or TCR+CD28 (46, 47). T cells lacking the p85 β isoform

proliferate normally in response to anti-CD3 plus anti-CD28 but show a surprising enhancement in cell division and survival when treated with anti-CD3 alone (+/- exogenous IL-2) (J. Deane, M. Trifilo, C. Yballe, S. Choi, T. Lane, D. Fruman, submitted manuscript). It seems reasonable to propose that p85 α and p85 β (and perhaps p50 α , which is also expressed in T cells) have redundant functions in mitogenic signaling, but this has not yet been tested in compound mutant mice.

The roles of catalytic isoforms p110 δ and p110 γ in T cell function have been somewhat controversial. In mice with a kinase-dead knock-in mutation in p110 δ , several aspects of T cell function are impaired, including Akt activation, Ca²⁺ mobilization, and anti-CD3-mediated proliferation (48). Although proliferation in response to co-crosslinking of CD3 and CD28 was not impaired in the absence of p110 δ , proliferation of TCR transgenic T cells stimulated by cognate antigen was reduced. Another group analyzing p110 δ -null mice reported normal T cell proliferation and Ca²⁺ mobilization, although the response to ConA was partially defective (50). It is possible that compensatory upregulation of p110 α and p110 β isoforms occurred in p110 δ -null T cells but not the p110 δ kinase-deficient T cells. Three groups disrupted the p110 γ gene and two reported defects in T cell development (13–15, 87). In one study, p110 γ -deficient mature T cells stimulated with anti-CD3 +/- anti-CD28 showed impaired proliferation, yet early signaling appeared to be intact (15). A plausible model is that p110 γ becomes activated via an as yet undefined GPCR and contributes to sustained PtdIns(3,4,5)P₃ production in T cells activated via the TCR. Similar mechanisms have been described in mast cells stimulated via Fc ϵ RI, monocytes stimulated with Fc γ RI, and fibroblasts stimulated with platelet-derived growth factor (88–90).

T cell proliferation is driven by the autocrine cytokine IL-2 that is produced in response to TCR engagement with appropriate costimulation. Like many cytokines, IL-2 is a potent activator of PI3K and Akt (2). IL-2-dependent events linked to the PI3K/Akt pathway include upregulation of Bcl-X_L, activation of E2F transcription factors, and increased S6 kinase activity (2). The mechanisms of PI3K activation by IL-2 and other mitogenic cytokines are reviewed in Reference (2).

There is conflicting evidence concerning whether PI3K is essential for IL-2 production. Wortmannin does not inhibit IL-2 production by murine T cells stimulated with anti-CD3 in the absence or presence of B7+ B cell blasts (91, 92), whereas another study found the opposite (93). When T cells are stimulated with APC+ peptide, PI3K has generally been found to be required for maximal IL-2 production (48, 92). Other early activation events are likely to be PI3K-independent. Recently, it was reported that LY294002 does not disrupt the immunological synapse nor prevent the upregulation of activation markers in T cells stimulated with APC+ peptide (81). Interestingly, the drug was completely effective at blocking cell division when added 9 h after mixing T cells with APC. Thus, T cells share with B cells a requirement for sustained PI3K activation to proceed through the cell cycle (76).

The Ca²⁺ mobilization response following antigen recognition by T cells is less dependent on PI3K than in B cells. PI3K inhibitors have little effect on Ca²⁺ flux

in response to APC-peptide (82, 94). A partial impairment in Ca^{2+} mobilization was reported in the p110 δ kinase-deficient T cells, but not in p110 δ -null cells (48, 50). The lack of a marked effect on the Ca^{2+} response is consistent with the observations that PI3K inhibitors fail to completely block IL-2 production or activation marker induction. A role for 3-phosphoinositides in aspects of Ca^{2+} mobilization cannot be fully discounted, especially given the novel observation that $\text{PtdIns}(3,4,5)\text{P}_3$ directly stimulates Ca^{2+} entry into T cells, but not B cells, via a membrane channel (95).

TCR-dependent Ca^{2+} mobilization does appear to require assembly of a signalosome analogous to the one in B cells, but with distinct components (2, 4) (Figure 4). T cells express Tec and Itk, Tec family kinases with PH domains homologous to the Btk PH domain. However, T cells also express Rlk, which is targeted to the membrane by palmitoylation and is presumably PI3K-independent. Rlk is essential for Ca^{2+} mobilization and subsequent functional responses in T cells that also lack Itk (96). The binding of Itk to $\text{PtdIns}(3,4,5)\text{P}_3$ is likely to be functionally significant as Itk is constitutively membrane-associated in Jurkat T cells (97), which have elevated basal $\text{PtdIns}(3,4,5)\text{P}_3$ levels due to the absence of PTEN and SHIP phosphatases (84). Interestingly, a null mutation in the Itk gene partially impairs Ca^{2+} mobilization, along with T cell proliferation and differentiation (98–100). The fact that PI3K inhibitors have little effect on Ca^{2+} mobilization whereas Itk is required, even with Rlk present, suggests that Itk has critical functions that do not require $\text{PtdIns}(3,4,5)\text{P}_3$ binding. It is worth considering whether Itk contributes to Ca^{2+} mobilization by associating with PIP5K, as demonstrated for Btk in B cells.

The importance of CD28 for physiological T cell activation and its defined role in PI3K activation has stimulated inquiry to understand the role of PI3K downstream of CD28. Several groups have approached this problem by expressing CD28 transgenes in the CD28-deficient background (101–103). These studies tend to support the conclusion that mutation of the Tyr residue in the Tyr-Met-Gln-Met motif impairs T cell survival and Bcl- x_L upregulation, but not IL-2 production. These defects cannot be attributed solely to impaired PI3K recruitment as Grb2 also interacts with this sequence. Nevertheless, other work has established that PI3K and Akt are important for CD28-dependent enhancement of NF κ B transcriptional function, and likely Bcl- x_L expression (72). A recent mechanistic advance in this regard was the identification of the MAP3K Cot1/Tp12 as an Akt substrate upstream of the IKK complex (104). Interestingly, costimulation via ICOS does not lead to upregulation of Bcl- x_L despite the greater activation of PI3K (86).

In apparent conflict with the finding that CD28 can costimulate production of IL-2 in the absence of PI3K recruitment (101–103), it was reported that expression of a constitutively membrane-targeted form of Akt could restore IL-2 production in CD28-deficient T cells (105). One possibility is that a high level of sustained Akt activation is required for increased IL-2 transcription, and this is provided by the membrane-targeted allele but not by regulated synthesis of 3-phosphoinositides downstream of CD28. An intriguing observation made more recently by the same

group (72) is that NF κ B activation by membrane-targeted Akt is dependent upon an intact PH domain. Thus, as in Btk, the PH domain of Akt appears to have critical functions in addition to mediating membrane recruitment.

The CD28/PI3K/Akt pathway may promote survival not only by upregulating Bcl-x_L, thus promoting mitochondrial integrity, but also via inhibition of death receptor signaling. A recent study found that CD28/Akt signaling blocked Fas-mediated apoptosis by preventing assembly of the death-inducing signaling complex (106). This mechanism is consistent with the finding that T cells lacking PTEN, or expressing constitutively active forms of PI3K or Akt, are resistant to Fas-mediated apoptosis (6).

A recent advance in the understanding of CD28 function was the finding that signaling via PI3K and Akt is essential for increased glucose transport, metabolism, and glycogen synthesis (107). These responses are essential for the ability of CD28 to costimulate proliferation and survival. The molecular link between Akt and altered metabolism is not yet established. The PI3K/Akt pathway has long been known to have an essential and evolutionarily conserved function in metabolic changes promoted by insulin and related growth factors (1). Akt-dependent phosphorylation and inactivation of FOXO transcription factors and GSK-3 are essential for metabolic responses in other systems, but it is not yet certain that these events contribute to CD28 costimulation. Of possible relevance, transgenic expression of constitutively active GSK-3 β impairs T cell proliferation (108). It has also been established that FOXO proteins are phosphorylated in a PI3K/Akt-dependent manner in activated T cells, and expression of an Akt-independent form of FOXO3a promotes cell cycle arrest and apoptosis in primary T cell blasts and IL-2-dependent clones (109). These effects have been linked to the FOXO target genes p27kip (a cell cycle inhibitor) and Bim (a proapoptotic protein) (Figure 4) rather than well-defined metabolic targets of FOXO such as phosphoenolpyruvate carboxylase kinase and glucose-6-phosphatase.

Cbl and Cbl-b are multifunctional proteins that are phosphorylated following BCR and TCR engagements and have complex roles in signal transduction (110). Cbl proteins have multiple protein-interaction motifs, including proline-rich and phosphotyrosine sequences that together recruit PI3K p85 subunits via their Src-Homology 3 (SH3) and SH2 domains. Cbl proteins also function as E3 ubiquitin ligases that promote degradation of other associated signaling proteins, including p85 (111). A negative role for Cbl proteins in antigen receptor signaling is suggested by the hyperproliferation observed in T cells lacking Cbl or Cbl-b, and B cells lacking Cbl-b (112, 113). Strikingly, loss of Cbl-b bypasses the requirement for CD28-mediated costimulation of T cell proliferation (114). However, these phenotypes do not appear to be the result of enhanced signaling protein stability (115). Cbl-b-deficient T cells have enhanced Vav activity (114), which could increase the efficiency of PI3K activation by TCR engagement alone. The understanding of Cbl protein function is further complicated by studies showing positive regulatory functions for Cbl in BCR-driven Ca²⁺ mobilization in DT40 cells and in CD40-dependent PI3K activation (116, 117). Elucidating the complex functions

of Cbl proteins will likely require the generation of knock-in mice with targeted mutations affecting individual interaction motifs.

IgE Receptor

Fc ϵ RI is the high-affinity IgE receptor that is central to allergic and inflammatory responses mediated by mast cells and basophils (27). Fc ϵ RI binds IgE and crosslinking by multivalent antigen triggers Ca²⁺ mobilization and other signaling pathways essential for degranulation and other responses. PI3K signaling contributes to Ca²⁺ mobilization and degranulation as well as to cytokine gene induction. Recent work has indicated that Fc ϵ RI crosslinking leads to association of PI3K with two signaling complexes that are spatially and functionally distinct in mast cells (118, 119). One complex is nucleated by tyrosine-phosphorylated Gab2. The other complex is analogous to the Ca²⁺ signalosomes of T cells and B cells and contains some of the same components, including Syk, LAT, Btk and Itk, SLP-76, Vav1, and PLC γ 1 (Figure 5). Assembly of this “LAT” complex requires Syk and the Src family kinase Lyn, whereas the Gab2 complex requires the Src family kinase Fyn (118).

Class IA PI3K regulatory subunits associate directly with tyrosine-phosphorylated Gab2. This interaction appears to be essential for Fc ϵ RI-mediated Akt

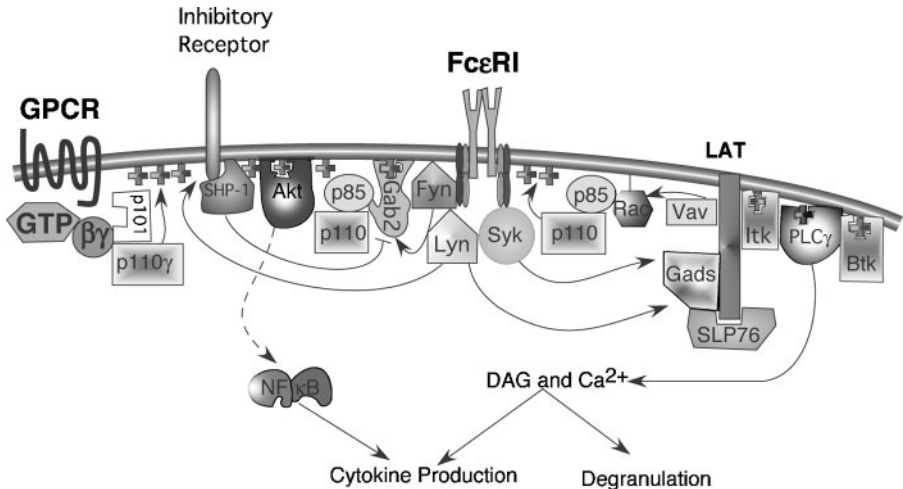


Figure 5 Role of PI3K in Fc ϵ RI signaling and amplification by GPCRs. The complexes containing either LAT or Gab2 are shown as physically separated, in keeping with recent findings. Also shown is an inhibitory receptor that recruits SHP-1 tyrosine phosphatase leading to Gab2 dephosphorylation; other inhibitory receptors on mast cells can recruit SHIP-1 as well. Note that Lyn can both promote mast cell activation (by phosphorylating signaling chains of Fc ϵ RI and components of the LAT complex) and oppose activation (by phosphorylating inhibitory receptor tails).

activation and allergic responses, based on studies of Gab2-deficient mast cells (120). The mechanism of PI3K association and activation in the LAT complex is not yet clear. PtdIns(3,4,5)P₃ production and Akt activation are attenuated in mast cells lacking Vav1 (121), suggesting that interaction with GTP-bound Rac proteins may contribute to PI3K activation, as reported in DT40 B cells (42). This model is consistent with a report that FcεRI-mediated Akt activation and Bcl-x_L upregulation are diminished in Rac2-deficient mast cells (122). One issue that remains to be resolved is why deletion of either Gab2 or Rac2, which presumably activate distinct pools of PI3K in spatially separated signaling complexes, each results in a near-blockade of Akt activation. One possibility is that PtdIns(3,4,5)P₃ acts as a diffusible second messenger to mediate crosstalk between the separate signaling complexes. In this view, small amounts of PtdIns(3,4,5)P₃ are produced initially by PI3K in the LAT complex, and the lipid then acts to recruit Gab2 to another membrane site via its PH domain (Figure 5). Subsequent Gab2 phosphorylation recruits more PI3K and amplifies PtdIns(3,4,5)P₃ production to a level necessary for Akt activation.

Ca²⁺ mobilization and degranulation can be attenuated by coligation of FcεRI with various inhibitory receptors expressed on mast cells. As in B cells, binding of IgE to antigen coated with IgG leads to coengagement of FcγRIIB1 and resultant recruitment of SHIP to the membrane and dephosphorylation of PtdIns(3,4,5)P₃ (123). Mast cells lacking SHIP have elevated basal and stimulated Ca²⁺-mobilization responses (25). Other inhibitory receptors may inhibit PI3K signaling by recruiting the tyrosine phosphatase SHP-1 (123), leading to dephosphorylation of critical signaling components such as Gab2. Recruitment of SHIP-1 or SHP-1 to inhibitory receptor cytoplasmic tails involves tyrosine phosphorylation of a sequence known as the ITIM (immunoreceptor tyrosine-based inhibitory motif). Phosphorylation of ITIMs by Lyn may play a central role in this feedback inhibition pathway (Figure 5), as Lyn-deficient mast cells have elevated basal Gab2 phosphorylation, PtdIns(3,4,5)P₃ levels, and Akt activity (124).

The roles of individual class IA PI3K isoforms in FcεRI signaling are not fully understood. Deletion of p85α/p55α/p50α does not impair FcεRI-dependent responses, including Akt phosphorylation (125). The same mast cells showed partial impairments in proliferation driven by Kit ligand, a transmembrane tyrosine kinase receptor with tandem Tyr-X-X-Met motifs. Mast cell function *in vivo* has not been studied in p85α/p55α/p50α knockouts because of the perinatal lethal phenotype of these mice. Mice lacking only p85α are viable and exhibit profound defects in mast cell function *in vivo*, but this has been attributed to defects in mast cell and T helper cell differentiation rather than a signaling defect in mature mast cells (3, 126). The phenotypes of mast cells lacking p85β or class IA catalytic subunits have not been reported. However, studies in which p110 isoform-specific antibodies were injected in the rat basophilic leukemia (RBL) cell line have suggested that p110α, p110β, and p110δ each have required functions in FcεRI-dependent degranulation, the latter two essential for Ca²⁺ mobilization (127, 128).

Class IB and class III PI3Ks can also regulate degranulation. The p110γ isoform is required for amplification of mast cell degranulation by GPCR ligands such as

adenosine (Figure 5) (90). This appears to be functionally important as p110 γ -deficient mice showed impairments in passive systemic anaphylaxis. Another study reported a novel function for class III PI3K in degranulation triggered by carbachol, a muscarinic receptor agonist (128).

The mechanisms by which 3-phosphoinositides promote Ca²⁺ mobilization and Akt activation downstream of Fc ϵ RI are probably similar to those described in the BCR and TCR sections. Btk and Itk are expressed in mast cells, and Btk-deficient mast cells show impaired responses to Fc ϵ RI crosslinking (129). One group studying mast cells has also reported that PtdIns(3,4,5)P₃ can directly stimulate PLC γ 1 activity (127), a finding supported by investigators working on nonhematopoietic cell types (130). PLC γ proteins possess two domains reported to interact with PtdIns(3,4,5)P₃: a PH domain and a SH2 domain, and lipid binding may increase enzyme activity by altering the tertiary structure of the protein and/or stabilizing membrane association. Whether these mechanisms are also involved in PI3K-dependent Ca²⁺ mobilization downstream of the BCR is not known. The role of Akt in Fc ϵ RI signaling has not been studied in as much detail as in BCR or TCR/CD28 systems. However, Akt has been linked to Bcl-xL upregulation in mast cells, as in other cell types (122).

Fc Receptors for IgG

Fc γ receptors (Fc γ Rs) are expressed in diverse hematopoietic cell types and mediate numerous cellular functions including phagocytosis and antibody-dependent cytotoxicity (28). Whereas the cytoplasmic tail of the inhibitory Fc receptor Fc γ RIIB1 contains an ITIM, the cytoplasmic tails of “activating” Fc γ R signaling chains contain ITAMs and initiate signaling mechanisms similar to those discussed above for the BCR, TCR, and Fc ϵ RI. In several cell types, crosslinking of Fc γ Rs has been linked to PI3K activation (2). Here we focus on Fc γ R-dependent phagocytosis, as notable recent advances have clarified the role of PI3K in this process.

PI3K activity is required for phagocytosis of large (>0.5 μ m) IgG-coated particles, with specific roles in pseudopod extension and contractile processes during phagosome closure (131–133). In the presence of PI3K inhibitors, smaller particles can be engulfed but phagosome maturation is impaired (134). Engulfment of large particles requires local expansion of plasma membrane, and PI3K activity is specifically required for exocytosis of membranes for this purpose (133). Fluorescence microscopy of cells expressing GFP fusion probes has been particularly informative in studies of 3-phosphoinositide dynamics during phagocytosis (134, 135). Consistent with a role in pseudopod extension and phagosome closure, PtdIns(3,4,5)P₃ accumulates in the nascent phagocytic cup and disappears shortly after the phagosome is sealed (Figure 6) (135). Interestingly, the 3-phosphoinositide species required for phagosome maturation is PtdIns(3)P, which appears in the phagosome membrane only after sealing (Figure 6). Immunofluorescent staining with antibodies to EEA1, an endosomal protein with a FYVE domain, and LAMP-1, a lysosome marker, was used to demonstrate impaired phagosome maturation in macrophages treated with wortmannin (Figure 6) (134).

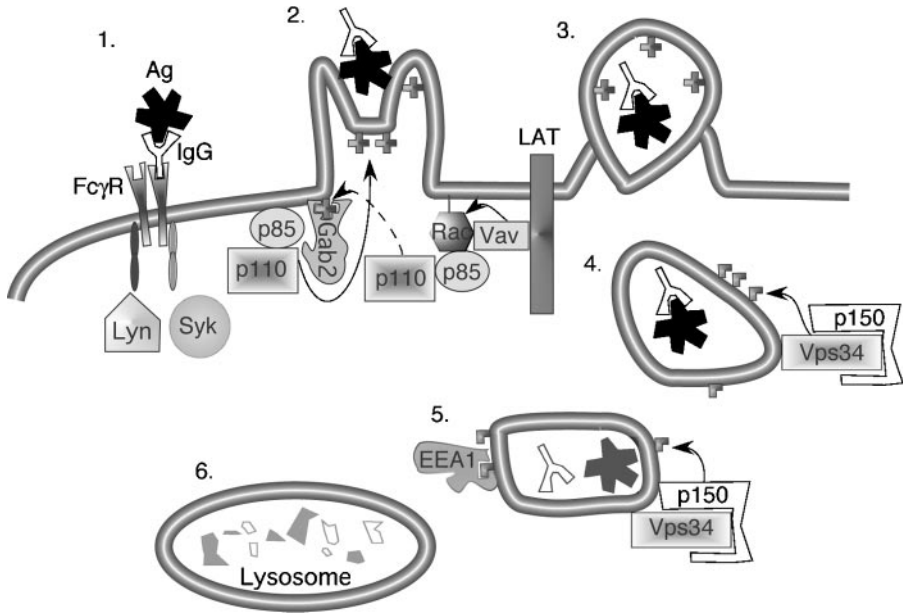


Figure 6 Role of PI3K in Fc γ R-mediated phagocytosis and phagosome maturation. Numbers represent temporal sequence of events. The initial activation of PI3K in the LAT complex downstream of Vav and Rac is speculative (see text). PtdIns(3)P in the membrane is represented by the inverted L-shapes.

Src family kinases and Syk are critical for tyrosine phosphorylation events that initiate Fc γ R signaling (2, 28). As in the Fc ϵ RI system, Gab2 contributes to activation of PI3K and Akt, and is required for Fc γ R-dependent phagocytosis (137). Gab2 is recruited to the phagocytic cup, a process that requires its PH domain and PI3K activity. Mutation of the p85 binding site on Gab2 reduces PtdIns(3,4,5)P₃ levels in the phagocytic cup. Thus, Gab2 is both downstream and upstream of PI3K, serving as an amplifier of 3-phosphoinositide production at a focused membrane site critical for phagocytosis. How does Fc γ R engagement trigger initial activation of PI3K required for Gab2 recruitment? As in mast cells, a complex involving LAT may be important. Class IA PI3K associates with tyrosine-phosphorylated LAT following Fc γ R crosslinking (138), and macrophages lacking LAT exhibit impaired phagocytosis (139). Vav is involved in Fc γ R-dependent Rac activation (140), but whether this is involved in PI3K activation is not known (Figure 6). Importantly, phagocytosis is enhanced in cells lacking Fc γ RIIB1 or SHIP, indicating that attenuating the PI3K pathway is a shared mechanism of inhibitory signaling in phagocytes as in mast cells and B cells (141).

Consistent with detection of PtdIns(3,4,5)P₃ in the phagocytic cup and PtdIns(3)P in sealed phagosomes, class I PI3K is essential for particle engulfment,

whereas class III PI3K is essential for phagosome maturation (Figure 6) (134). In this report, class IA isoforms were shown to contribute to phagocytosis in experiments in which fibroblasts lacking most regulatory isoforms (p85 α /p55 α /p50 α /p85 β) were transfected with Fc γ RIIA. Compared with wild-type fibroblast transfectants, PtdIns(3,4,5)P₃ production and engulfment of large particles was significantly impaired. Specific roles for the class IA catalytic isoform p110 β in phagocytosis, and the mammalian class III PI3K (VPS34) in phagosome maturation, were demonstrated recently by microinjection of specific antibodies (134, 142, 143). As in mast cells stimulated via Fc ϵ RI, PtdIns(3,4,5)P₃ production in monocytes stimulated via Fc γ RI is amplified by activation of the class IB isoform p110 γ (88).

The PtdIns(3,4,5)P₃-binding proteins that mediate exocytosis of intracellular membrane reserves have not been established, but may include regulators of the small G protein Arf6 (10, 144). 3-Phosphoinositides have defined roles in actin cytoskeletal remodeling events involved in membrane ruffling and directional movement (see below), yet PI3K appears to be dispensable for actin polymerization during phagocytosis (133, 134). However, an atypical form of myosin that contains a PH domain was recently implicated as a link between PtdIns(3,4,5)P₃ and pseudopod extension (145).

PI3K IN OTHER LEUKOCYTE RECEPTOR SIGNALING SYSTEMS

ITAM/ITIM-Containing Receptors on NK Cells

Natural killer (NK) cells are important for the innate immune response to viruses and tumor cells, yet share many attributes with effector cells of the adaptive immune response (146). NK cell activation is based on the integration of signals from activating receptors containing ITAMs and inhibitory receptors containing ITIMs (147). As for inhibitory receptors in other cell types, ITIM sequences in NK inhibitory receptors can recruit SHIP1 as well as SH2-containing tyrosine phosphatases (148, 149). An important role for 3-phosphoinositides in NK cell development and function is supported further by the finding that mice lacking SHIP1 have a marked increase in NK cells, combined with a decrease in NK-mediated bone marrow graft rejection (148).

Several NK-activating receptors share the ability to activate PI3K, including NKG2D, CD28, and 2B4 (reviewed in 150). We focus here on NKG2D, whose signaling mechanisms have been clarified recently. NKG2D associates with two adapter proteins, DAP10 and DAP12, which mediate assembly of distinct signaling complexes (151). DAP10 possesses a Tyr-Ile-Gln-Met motif that can interact with class IA PI3K, whereas DAP12 possesses ITAMs that are linked to activation of Syk and ZAP-70 (151, 152). Recent studies of both human and mouse NK cells have indicated that DAP12 is primarily responsible for triggering cytokine production, whereas DAP10 is important for cytotoxicity downstream of NKG2D (153, 154).

Cytotoxicity is blocked by LY294002 or mutation of the Tyr-Ile-Gln-Met motif in DAP10, emphasizing the importance of PI3K activation in the DAP10 complex.

Receptors for Chemoattractants

Regulated synthesis and localization of 3-phosphoinositides play an evolutionarily conserved role in directional movement of cells exposed to gradients of chemoattractants (155, 156). PI3K inhibitors inhibit leukocyte chemotaxis induced by microbial products (e.g., fMLP), inflammatory products (e.g., C5a), and numerous chemokines. Fluorescence imaging of cells expressing GFP-PH fusion probes has revealed a striking concentration of PtdIns(3,4,5)P₃ at the leading edge of migrating neutrophils (157), and there is considerable evidence that 3-phosphoinositides help determine cell polarity during directional movement (10).

Recent work has provided new mechanistic insights into how PtdIns(3,4,5)P₃ is concentrated at the leading edge of migrating cells, even in shallow gradients of chemoattractant (158, 159). These studies took advantage of two methodological advances: detection of PtdIns(3,4,5)P₃ with a GFP-PH fusion probe, and the ability to deliver exogenous PtdIns(3,4,5)P₃ to the cytoplasmic leaflet of the membrane using cationic vesicles. Using these techniques, it was shown that PtdIns(3,4,5)P₃ delivery to neutrophils induces a positive feedback loop leading to production of more PtdIns(3,4,5)P₃ by endogenous PI3K. The mechanism involves PtdIns(3,4,5)P₃-dependent activation of Rho family G proteins, which in turn amplify PI3K activity (Figure 7). Interestingly, actin polymerization at the leading edge, which requires PtdIns(3,4,5)P₃ and Rho family G proteins, also plays a role in the positive feedback loop by helping to maintain proper polarity in PtdIns(3,4,5)P₃ accumulation (159).

The Rac subfamily of Rho G proteins is probably involved in the feedback amplification loop. Recently, the RacGEF pRex-1 was purified from neutrophil extracts based on its ability to mediate PtdIns(3,4,5)P₃-dependent GTP loading of Rac (160). pRex-1 has a PH domain but this has not yet been demonstrated to mediate the effects of PtdIns(3,4,5)P₃. Importantly, pRex-1 activation is also promoted by G protein $\beta\gamma$ subunits, and antisense inhibition of pRex-1 expression inhibits Rac-dependent superoxide production induced by C5a. These findings support the model that pRex-1 is a critical mediator of PtdIns(3,4,5)P₃-dependent Rac activation downstream of GPCRs (Figure 7) (161).

Most chemoattractant receptors are members of the GPCR superfamily. Thus, it is not surprising that the sole class IB PI3K catalytic isoform p110 γ is required for PtdIns(3,4,5)P₃ production and Akt activation downstream of the receptors for fMLP, C5a, and IL-8 (13–15). Mice lacking p110 γ show reduced neutrophil and macrophage chemotaxis *in vitro* and impaired leukocyte recruitment to inflammatory sites *in vivo*. p110 γ -deficient neutrophils show impaired Rac activation and F-actin accumulation at the leading edge (14, 162). Class IA PI3K can also be activated by GPCRs and contribute to chemotaxis (162a). A specific role for p110 δ in neutrophil chemotaxis to GPCR ligands was recently demonstrated using a novel

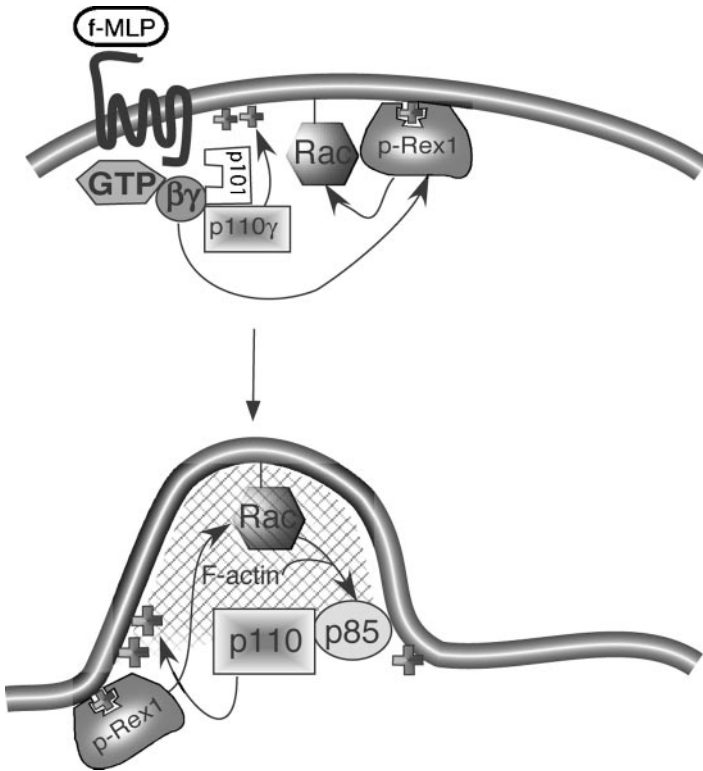


Figure 7 Role of PI3K in chemotaxis to GPCR ligands (fMLP shown in model). In this two-step model, initial activation of class IB PI3K synergizes with $G_{\beta\gamma}$ subunits to activate the Rac exchange factor pRex-1. Subsequent Rac activation and actin polymerization (*hatched area*) contribute to feedback amplification of $\text{PtdIns}(3,4,5)\text{P}_3$ production, in part via class IA PI3K isoforms. This could be mediated directly via a Rac-class IA PI3K interaction, via recruitment of PIP5K to generate more substrate, or through other mechanisms such as polarized accumulation of tyrosine kinases that are transactivated by GPCR signaling.

inhibitor specific for this class IA isoform (163). Notably, F-actin synthesis was not blocked. Thus, a plausible model is that $p110\delta$ is involved in the Rac- and F-actin-dependent feedback amplification loop, whereas $\text{PtdIns}(3,4,5)\text{P}_3$ produced by $p110\gamma$ cooperates with $G_{\beta\gamma}$ subunits to initiate actin polymerization via pRex-1 and Rac activation (Figure 7). Class IA catalytic isoforms are also involved in chemotaxis of macrophages to colony stimulating factor, whose receptor possesses intrinsic tyrosine kinase activity (164).

Certain chemotactic ligands (e.g., fMLP, C5a) also trigger neutrophils to release reactive oxygen species via the activation of a complex known as the NADPH

oxidase. This response, also known as oxidative burst, requires activation of class IB PI3K, pRex-1, and Rac (13–15, 160, 165). Interestingly, priming of neutrophils with TNF α or GM-CSF, which greatly enhances fMLP-mediated oxidative burst, correlates with enhanced production of PtdIns(3,4,5)P₃ via p110 γ (166). In addition to the indirect role of 3-phosphoinositides in NADPH oxidase activation via pRex-1 and Rac, PtdIns(3)P and PtdIns(3,4)P₂ bind directly to cytosolic components of the complex to facilitate assembly of an active complex at the phagosomal membrane (reviewed in 2, 18).

Lymphocyte migration within lymphoid organs and to sites of infection and inflammation is controlled by a variety of chemokines that act via GPCRs. The mechanisms by which PI3K contributes to lymphocyte chemotaxis are less well studied than those in neutrophils and macrophages. The p110 γ isoform seems likely to play a role, but this has not been directly tested. However, defects in T and B cell development observed in p110 γ -deficient mice (14, 15) may be the result of impaired migration. A role for Rac proteins in lymphocyte chemotaxis is supported by the finding of impaired chemokine-induced migration of T and B cells lacking Rac2 (54, 167). However, it is not yet clear whether PI3K acts upstream and/or downstream of Rac in lymphocyte chemotaxis. It has been reported that B cells lacking one or both copies of the PTEN gene show enhanced chemotaxis in response to a number of chemokines (78, 168). On the other hand, another group found impaired chemotaxis in PTEN-deficient B cells despite increased basal Rac activation (33). Although the reason for the conflicting results is not clear, it is important to note that PTEN in *Dictyostelium* has an essential role in directional migration by metabolizing PtdIns(3,4,5)P₃ at the trailing edge of the cell (155). These findings emphasize that augmented PtdIns(3,4,5)P₃ production can promote chemotaxis only when produced at a focal area of the cell.

PERSPECTIVES

PI3K activation is a common signal transduction event in a remarkable variety of functional responses in leukocytes. How does production of 3-phosphoinositides promote different responses downstream of distinct receptors? As emphasized throughout this review, an important mechanism is that PI3K effector proteins must integrate signals from lipid binding and other intermolecular interactions in order to be recruited to signaling complexes and activated. Thus, the spectrum of PI3K effectors engaged by distinct receptors differs. Examples include synergistic activation of pRex-1 by PtdIns(3,4,5)P₃ and G $\beta\gamma$ downstream of GPCRs, and activation of Tec family tyrosine kinases by membrane-associated Src family kinases that are activated by ITAM-containing receptors. Feedback amplification loops further enhance localized synthesis of 3-phosphoinositides, augmenting and sustaining the activation of PI3K effectors at focal areas of the cell where dynamic changes in membrane dynamics occur. Examples include Gab adapter-based amplification (Figure 5 and 6) and the Rac-mediated feedback loop in chemotaxis (Figure 7).

A paradigm has also emerged in antigen and Fc receptor systems in which receptor occupancy leads to the assembly of large macromolecular complexes containing PI3K, its lipid products, along with PI3K activators and effectors (Figures 3–6). In these large complexes or signalosomes, information is exchanged among components and the distinctions between upstream and downstream events can be obscured. Removal of any component can disrupt function of the entire complex. An additional level of organization is probably provided by the localization of antigen/Fc receptor signaling complexes in glycolipid-enriched membrane microdomains, also known as lipid rafts. Although not emphasized in this review, lipid rafts clearly play an important role in assembly of signalosomes; conversely, receptor signaling can promote aggregation of lipid rafts (169, 170). There is growing evidence for a relationship between PI3K signaling and lipid raft aggregation. In B cells, recruitment of SHIP to lipid rafts by the inhibitory receptor Fc γ RIIB1 inhibits raft aggregation (171). In T cells lacking p110 δ , raft aggregation is impaired following cocrosslinking of CD3 and CD28 (48). Clearly, there is much still to learn about the interplay between lipid rafts, PI3K and signalosome assembly.

Other questions worthy of further investigation include: How does the composition of membrane-associated signalosomes differ downstream of different receptors, especially those less well studied than the BCR and TCR? What are the unique functions of PI3K isoforms in various signaling complexes, and do these functions change at different times during development? Which modular domains of PI3K catalytic and regulatory isoforms are critical for enzyme activation in different contexts? How does the role of PI3K signaling differ at distinct points during cell cycle progression of activated lymphocytes? New methodological tools are helping to address these questions more easily under physiological conditions. Given the heterogeneity of primary cells, it will be especially important to study signal transduction at the single-cell level, using fluorescence-based technologies such as confocal microscopy and flow cytometry (34).

When reviewing the literature on PI3K, an easier task would perhaps be to list the few responses that are not dependent on this ubiquitous signaling pathway. Here we have noted a few instances in which a particular biological response can be either PI3K-dependent or -independent, depending on cell type or receptor system. For example, actin polymerization is regulated by PI3K signaling during chemotaxis but appears to be PI3K-independent during pseudopod extension and phagocytosis. Ca²⁺ mobilization is clearly influenced by PI3K following BCR crosslinking, but apparently less so following TCR engagement. Further detailed analysis of which biological responses require PI3K activation, and which PI3K isoforms and effector proteins are involved, will eventually allow predictions to be made about components within the pathway that could be viable pharmacological targets for different disease states. Already a compound has been developed that inhibits p110 δ , a leukocyte-specific PI3K catalytic isoform, and shown to impair neutrophil migration (163). It is hoped that continued efforts to define specificity in PI3K signaling will uncover opportunities to manipulate the pathway for therapeutic benefit (172).

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NOTE ADDED IN PROOF

Strong evidence that Rac proteins promote BCR-mediated PI3K activation has been provided by a study of mice with B cell-specific deletion of Rac1 and Rac2 (173). In addition, a study of Bam32-deficient mice has implicated this PH domain-containing protein in linking BCR-mediated PI3K activation to the Erk pathway (174).

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