PI-3 Kinase in Signalling and Disease

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Towards an understanding of isoform specificity in phosphoinositide 3-kinase signalling in lymphocytes

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Abstract

The PI3K (phosphoinositide 3-kinase) signalling pathway promotes proliferation and transformation in many cell types. This is particularly well illustrated by studies of primary lymphocytes and their leukaemic counterparts. PI3K activation is required for proliferation of T cells and B cells, and certain oncogenes cause leukaemia in part by promoting PI3K signalling. Genetic manipulation of this pathway, together with biochemical studies in primary lymphocytes, has begun to shed light on the molecular mechanisms by which PI3K contributes to the proliferation of normal and transformed lymphocytes. In particular, targeted gene disruption in mice has allowed the identification of specific isoforms of PI3K that are required for distinct cellular responses. Continued investigation of isoform specificity in PI3K signalling, as well as the characterization of critical downstream targets of PI3K signalling, may reveal strategies for the therapeutic control of immune responses and leukaemia.

Overview of PI3K (phosphoinositide 3-kinase)

(For more detailed reviews, see [1,2].) PI3K enzymes are lipid kinases that phosphorylate the 3-hydroxy group of phosphatidylinositol and related inositol phospholipids to generate products known as 3-phosphoinositides. These lipids act at local membrane sites to recruit cytoplasmic proteins and help assemble signalling complexes. There are eight known PI3K catalytic isoforms in mammals (Table 1). These are divided into four classes: class IA, class IB, class II and class III. These differ in substrate selectivity and regulation. Only the class IA and IB enzymes are able to generate PtdIns(3,4,5)P_3, a critical second messenger. Class IA enzymes are activated primarily by tyrosine kinase-initiated signalling pathways, whereas class IB enzymes function mainly downstream of GPCRs (G-protein-coupled receptors). PH (pleckstrin homology) domains are the most common protein module known to mediate PtdIns(3,4,5)P_3-dependent membrane recruitment.

There are three genes encoding class IA catalytic isoforms. Each encodes a protein product of approx. 110 kDa, denoted p110α, p110β and p110δ. These form stable heterodimers with class IA regulatory subunits, of which at least five isoforms exist (p85α, p55α, p50α, p85β and p55γ). p85α, p55α and p50α are alternative transcripts of a single gene, *Pik3r1*, whereas p85β and p55γ are encoded by distinct genes. Class IA PI3K heterodimers are mainly cytoplasmic in unstimulated cells, but a fraction translocates to cellular membranes in response to extracellular ligands that trigger activation of tyrosine kinases. Recruitment to membrane signalling complexes serves not only to localize the enzyme in the vicinity of its substrates but also to increase intrinsic activity via allosteric interactions. Modular domains within the regulatory subunits, particularly the two SH2 (Src homology 2) domains, are primarily responsible for association with phosphotyrosine-containing complexes. Most class IA catalytic and regulatory isoforms have broad and overlapping tissue distributions. An apparent exception is p110δ, which is found mainly in leucocytes.

There is a single class IB enzyme, p110γ. This associates with a unique regulatory subunit termed p101. Together this dimer is sometimes called PI3K γ. Activation of PI3K γ

Key words: B cell, isoform specificity, leukaemia, lymphocyte, phosphoinositide 3-kinase (PI3K), T cell.

Abbreviations used: APC, antigen-presenting cell; BCR, B cell receptor; Btk, Bruton's tyrosine kinase; FOXO, Forkhead Box, subgroup O; GPCR, G-protein-coupled receptor; NFκB, nuclear factor κB; PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; PTEN, phosphatase and tensin homologue deleted on chromosome 10; TI-2, T-independent type II.

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Table 1 | Phenotypes of mice with disrupted PI3K genes

Gene product(s)	PI3K class/type	Summary of phenotypes in lymphocytes	References
p110α	IA catalytic	Not determined; embryonic lethal	[24]
p110β	IA catalytic	Not determined; embryonic lethal	[25]
p110δ	IA catalytic	Reduction in mature B cells; absence of B1 and MZ (marginal zone) B cells; impaired proliferation to B cell mitogens; diminished antibody responses; impaired T cell proliferation to anti-CD3 or APCs + peptide	[5–7]
p110γ	IB catalytic	Altered Ig light chain ratios; increased thymocyte apoptosis; impaired T cell proliferation to anti-CD3; impaired T cell responses *in vivo*	[26,28]
p85α, p55α and p50α	IA regulatory	Reduction in mature B cells; absence of B1 cells; impaired proliferation to B cell mitogens; antibody responses not tested	[3]
p85α	IA regulatory	Reduction in mature B cells; absence of B1 cells; impaired proliferation to B cell mitogens; diminished antibody responses; B cell development defects overcome by Bcl-XL transgene	[4,14]
p85β	IA regulatory	Enhanced T cell proliferation and survival to anti-CD3	[23]

downstream of GPCRs is mediated by $\beta\gamma$ complexes from heterotrimeric G-proteins. PI3K γ is most abundant in leucocytes.

The yeast genome encodes only a class III enzyme, and invertebrate genomes generally encode a single isoform of classes IA, II and III, usually with no class IB PI3K. Thus mammalian evolution clearly has been accompanied by duplication and diversification of PI3K functions. One hypothesis is that the adaptive immune system employs complex regulatory circuits that have driven evolution of PI3K diversity in vertebrates. To address how individual class I PI3K isoforms function in the immune system, several laboratories have used a gene targeting approach in mice. This review will summarize the findings from these experiments, while mentioning some previously unpublished work that was described at the Biochemical Society Focused Meeting on 'PI 3-Kinase in Signalling and Disease'.

PI3K isoforms and downstream targets in B cell development and activation

The p85α regulatory subunit and the p110δ catalytic subunit of class IA PI3K have essential roles in the development and activation of murine B cells. The *Pik3r1* gene has been disrupted in two ways, one in which all three protein products are eliminated (here termed *Pik3r1 null*) and one in which only p85α is removed and p55α and p50α can still be expressed (here termed p85αKO). Similar defects in B cell development and function are seen in these two mouse strains [3,4] and in three independent strains of mice with targeted inactivation of the gene encoding p110δ [5–7] (Table 1). There is a partial block in early B cell development at the pro-B to pre-B transition, a marked reduction in the number of mature splenic B cells, and a nearly complete absence of the B1 subset of mature B cells. The few B cells that can be isolated from spleens of p85α-deficient or p110δ-deficient mice fail to proliferate following clustering of the BCR (B cell receptor) with anti-IgM. Proliferation is also impaired in response to the polyclonal B cell mitogens lipopolysaccharide and anti-CD40. p85αKO mice and mice lacking p110δ also fail to mount effective antibody responses to TI-2 (T-independent type II) antigens.

These phenotypes are remarkably similar to those observed in a growing number of mouse strains lacking key signalling proteins expressed in B cells (reviewed in [2,8,9]). The list of proteins required for efficient B cell development, proliferation and TI-2 responses includes the tyrosine kinase Btk (Bruton's tyrosine kinase), the adaptor proteins BLNK (B cell linker protein) and BCAP, the B cell co-receptor CD19, the guanine nucleotide exchange factor Vav (Vav1 and Vav2 are partially redundant), the small GTPase Rac2 and PLCγ2 (phospholipase Cγ2) [2,8–10]. Another common defect in B cells lacking these proteins is a diminished Ca^{2+} mobilization response following BCR engagement. Impaired Ca^{2+} mobilization is also observed in B cells lacking p110δ or p85α ([5–7]; K.L. Hess, A.C. Donahue and D.A. Fruman, unpublished work). These genetic correlations, together with established biochemical interactions, have led to the 'signalosome' model of BCR signalling, in which a large group of signalling proteins assemble and work together to promote maximal PLCγ2 activation [8]. This partnership allows sufficient Ins(1,4,5)P_3 to be produced to drive Ca^{2+} mobilization. A critical downstream effector of the signalosome, which integrates inputs from both Ca^{2+} and diacylglycerol, is PKCβ (protein kinase Cβ). Loss of PKCβ abolishes BCR-dependent proliferation [11]. Recent evidence indicates that PKCβ provides a crucial link between the BCR signalosome and activation of NFκB (nuclear factor κB)-dependent transcription [12–15].

The role of PI3K in the signalosome is thought to be to properly assemble the complex at the plasma membrane near the BCR and tyrosine kinases of the Src and Syk families. Full activation of PLCγ2 requires at least four events: recruitment to the membrane to gain access to its substrate PtdIns(4,5)P_2, phosphorylation by Syk, phosphorylation by Btk, and maintenance of local PtdIns(4,5)P_2 levels. Each of these steps may be regulated by PI3K. Both Btk and PLCγ2 possess modular lipid-binding domains that are implicated in

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PtdIns(3,4,5)P_3-dependent membrane recruitment [16–18]. In early versions of the signalosome model, it was proposed that Btk activation and subsequent phosphorylation of PLCγ2 is PtdIns(3,4,5)P_3-dependent [8]. This supposition was based on a number of observations, including the fact that Btk function can be essentially abolished by a point mutation in the PH domain that abrogates PtdIns(3,4,5)P_3 binding. However, some data have accumulated suggesting that triggering of Btk kinase activation downstream of the BCR is PtdIns(3,4,5)P_3-independent. The main piece of evidence for this claim is a report that Btk kinase activity is normal in B cells lacking p85α or treated with global PI3K inhibitors [14]. A subsequent study provided a potential resolution by showing that PtdIns(3,4,5)P_3 can work through Btk to help re-supply the PLCγ2 substrate [19]. Specifically, it was reported that Btk associates with PtdIns4P 5-kinase, an enzyme that can generate PtdIns(4,5)P_2. PtdIns4P 5-kinase expression enhanced the ability of PI3K and Btk to promote Ca^{2+} mobilization. Thus these findings support a modified signalosome model in which an important function of PtdIns(3,4,5)P_3-dependent membrane recruitment is to allow shuttling of PtdIns4P 5-kinase to the site where PLCγ2 (and potentially PI3K itself) is actively depleting local PtdIns(4,5)P_2.

PI3K activation in B cells does not merely promote signalosome assembly and activation of PKC and the NFκB pathway. Production of PtdIns(3,4,5)P_3 also triggers activation of Akt, a PH domain-containing serine/threonine kinase that acts downstream of PI3K in most cell types examined. Akt phosphorylates a large number of substrates with important roles in the control of cell growth, proliferation and survival. It is not yet clear which of the potential Akt substrates are relevant for B cell activation. It is likely that Akt-dependent phosphorylation of FOXO (Forkhead Box, subgroup O) proteins promotes cell cycle progression in primary B cells. The FOXO family of transcription factors activate target genes involved in cell cycle arrest, quiescence and apoptosis [20,21]. PI3K/Akt-dependent FOXO phosphorylation inactivates these factors and promotes their nuclear export. We find that this paradigm holds true in BCR-activated murine B cells as well (I. Yusuf, X. Zhu, M.G. Kharas and D.A. Fruman, unpublished work). Supporting a functional role for PI3K-dependent FOXO inactivation, forced expression of a mutant FOXO that cannot be phosphorylated by Akt promotes cell cycle arrest and apoptosis in primary B cells (I. Yusuf, X. Zhu, M.G. Kharas and D.A. Fruman, unpublished work).

Several class IA catalytic and regulatory isoforms are expressed in B cells, yet loss of p110δ alone or p85α alone has dramatic effects on B cell development and function. Proliferation in response to various mitogens is impaired in p85α-deficient mice to a similar extent as in wild-type cells treated with PI3K inhibitors [3]. This suggests that complexes of p110δ with p85α are responsible for most of the PI3K signalling output in B cells. This conclusion is supported by one study of p110δ-deficient B cells in which PtdIns(3,4,5)P_3 production, as measured by a novel *in vitro* FRET (fluorescence resonance energy transfer) assay, was abolished following BCR engagement [6]. Phosphorylation of Akt, often used as a read-out of PI3K signalling output, was also reported to be greatly diminished in B cells lacking either p85α or p110δ [5,6,14]. Deletion of the p85β gene does not appreciably alter B cell development, activation or antibody production (J.A. Deane, M.J. Trifilo, C.M. Yballe, S. Choi, T.E. Lane and D.A. Fruman, unpublished work) (Table 1). Whether p110α or p110β have unique roles in B cells (or other immune cell types) has been difficult to assess, as germline deletion of the genes encoding these isoforms results in early embryonic lethality [24,25] (Table 1). The class IB isoform p110γ appears dispensable for most aspects of B cell development and function [26–28] (Table 1).

PI3K isoforms and downstream targets in T cell development and activation

Class I PI3K is activated following engagement of the T cell receptor and augmented by co-stimulation via CD28 and other receptors on T cells [29]. A functional role for PI3K is suggested by the finding that global PI3K inhibitors block the proliferation of primary T cells in most experimental systems examined. Physiological T cell activation is accompanied by rapid and sustained production of PtdIns(3,4,5)P_3 that is concentrated at the site of contact with APCs (antigen-presenting cells). This was shown in elegant microscopy studies in which a fusion protein of the Akt PH domain with green fluorescent protein was expressed as a transgene in T cells and used to monitor PtdIns(3,4,5)P_3 production in live cells [30,31]. However, no single PI3K catalytic or regulatory isoform has been demonstrated to be absolutely essential for T cell development or proliferation.

T cells in *Pik3r1* null mice or p85αKO mice develop normally and proliferate in response to various mitogens [3,4]. In mice lacking p85β, T cell development and proliferation are also unimpaired (J.A. Deane, M.J. Trifilo, C.M. Yballe, S. Choi, T.E. Lane and D.A. Fruman, unpublished work). Surprisingly, p85β-deficient T cells exhibit enhanced cell division and survival *in vitro* and *in vivo* (J.A. Deane, M.J. Trifilo, C.M. Yballe, S. Choi, T.E. Lane and D.A. Fruman, unpublished work) (Table 1). The molecular mechanism of enhanced proliferation is not yet clear; however, an intriguing observation is that up-regulation of caspase-6, which may be involved in apoptosis of activated T cells, is diminished in activated p85β-deficient T cells. It is possible that p85α and p85β have redundant functions in T cell activation, with p85β having an additional role in a signalling pathway that sensitizes cells to death.

Two groups that deleted the gene encoding p110δ reported no defects in T cell development or function [6,7]. Another group used a 'knock-in' approach to introduce a point mutation that inactivates p110δ kinase activity, and observed defects in T cell proliferation *in vitro* [5]. Interestingly, proliferation driven by APCs plus antigen or anti-CD3 antibodies was impaired in the p110δ kinase-dead T cells, whereas the response to anti-CD3 plus anti-CD28

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was normal (Table 1). Biochemical analysis of anti-CD3-stimulated T cells revealed impaired Ca^{2+} flux and activation of ERK (extracellular-signal-regulated kinase) along with markedly reduced phosphorylation of Akt. Of note, proliferation defects in p110δ kinase-dead T cells were less dramatic than in B cells from these mice. It was reported that p110γ, the GPCR-activated isoform, is essential for optimal T cell proliferation [28] (Table 1), although this was not observed in all strains of p110γ-deficient mice [26,27]. It is possible that autocrine factors produced by T cells are GPCR ligands that can augment PI3K activation, a paradigm described previously in other immune cell types. More work needs to be done to clarify the unique and overlapping roles of class IA and IB PI3K isoforms in T cell activation.

It is important to consider that PI3K may play a fundamentally different role in the activation of T cells compared with B cells. PI3K inhibitors have little effect on Ca^{2+} mobilization in T cells activated by APCs plus antigen [31]. Furthermore, global PI3K inhibition impairs up-regulation of activation markers in B cells but not T cells [30,32]. Proliferation of T cells stimulated with APCs plus antigen can be blocked by addition of inhibitors as late as 9 h after formation of T-cell–APC conjugates [30]. Together with the finding that PtdIns(3,4,5)P_3 production is sustained for several hours following antigen recognition by T cells, these findings indicate that a major role of PI3K in T cells may be to drive cell cycle progression by autocrine growth factors. It is certainly well established that interleukin-2 and other cytokines activate PI3K and drive proliferation and survival in a manner dependent on PI3K [33].

What are the critical PI3K effectors in T cells? Although Btk is not expressed, other members of this family are expressed in T cells. One of these, Rlk, is tethered to the membrane by palmitoylation and does not contain a PH domain [34]. The ability of Rlk to promote Ca^{2+} flux could explain the failure of PI3K inhibitors to attenuate this response in T cells. It is likely that Akt plays a crucial role in transmitting PI3K signals to T cell survival and proliferation [29]. This is supported by studies of transgenic mice in which activated forms of Akt expressed in T cells cause enhanced proliferation and resistance to apoptosis [35].

PI3K isoforms and downstream targets in leukaemic transformation

Many neoplasms exhibit enhanced PI3K signalling. The molecular mechanisms for this are diverse, and include expression of oncogenes that activate PI3K, as well as PI3K gene amplification and mutations/deletions in class IA regulatory subunits [36]. Most commonly, tumour cells have lost function of the tumor suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10), a lipid phosphatase that directly opposes PI3K signalling [36]. Deletion of PTEN in T cells or B cells enhances their proliferation [37–39], and mice heterozygous for PTEN show a propensity for leukaemia and lymphoma [40–42]. Can specific PI3K isoforms be linked to leukaemia?

We have addressed this question using the model of murine pre-B cell transformation by oncogenic forms of Abl tyrosine kinase. PI3K has been shown to contribute to myeloid transformation by the p210 form of the human fusion oncogene BCR–Abl. Similarly, we find that global PI3K inhibitors block pre-B cell transformation by the p190 isoform of BCR–Abl or the Abelson virus oncogene v-Abl [43]. However, loss of p85α or p85β does not impair v-Abl-mediated transformation *in vitro*. Transformation by p190-BCR–Abl, as well as leukaemogenesis *in vivo*, are only modestly impaired in *Pik3r1* null cells. Thus there appears to be redundancy in the functions of class IA regulatory isoforms in murine pre-B cell transformation by Abl oncogenes. In contrast, antisense inhibition of p85α expression was reported to inhibit transformation of human haematopoietic cells by p210-BCR–Abl [44].

Activation of Akt is considered to be an underlying cause of transformation in many tumour cells with elevated PI3K signalling output [36]. Of relevance to lymphocytes, Akt was originally cloned as the transforming oncogene of a virus that causes thymomas; in this virus, the protein is fused to viral Gag protein sequences that cause constitutive membrane targeting and activation of the kinase [45]. Akt is required for myeloid transformation by p210-BCR–Abl [46] and is likely to mediate some of the oncogenic properties of Abl oncogenes in pre-B cells. This is consistent with our finding that FOXO proteins are highly phosphorylated in pre-B cells transformed by either v-Abl or BCR–Abl [43]. Inhibitors of PI3K or Abl kinase reduce FOXO phosphorylation. Furthermore, overexpression of PI3K-independent FOXO causes cell cycle delay and substantial apoptosis in these cell lines.

Perspectives

Early studies of PI3K revealed that this lipid kinase regulates an extraordinary number of cellular responses downstream of a vast array of receptors. Subsequent work has attempted to define how the PI3K signal is decoded to produce the specific response that is needed. One ultimate goal of these studies is to determine if therapeutic targets in the signalling pathway can be identified [47]. Are there PI3K isoforms or downstream effectors that function in specific immune processes or pathogenic scenarios? The identification of Btk as a critical PI3K effector suggests that these efforts are beginning to bear fruit, as Btk is a tyrosine kinase whose expression is restricted to B cells and a few other haematopoietic cell types. The leucocyte-specific catalytic isoform p110δ is involved in the function of both T and B cells. p110γ is a promising target for anti-inflammatory therapies, because neutrophils and macrophages from p110γ-deficient mice show markedly reduced responses to GPCR ligands [26–28]. Further work on PI3K signalling specificity is likely to uncover more interesting biology and ideas for pharmacology.

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