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PIP3: Tool of Choice for the Class I PI 3-kinases

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Abstract

Class I PI 3-kinases signal by producing the signaling lipid phosphatidylinositol (3,4,5) trisphosphate, which in turn acts by recruiting downstream effectors that contain specific lipid-binding domains. The Class I PI 3-kinases comprise four distinct catalytic subunits linked to one of seven different regulatory subunits. All the Class I PI 3-kinases produce the same signaling lipid, PIP3, and the different isoforms have overlapping expression patterns and are coupled to overlapping sets of upstream activators. Nonetheless, studies in cultured cells and in animals have demonstrated that the different isoforms are coupled to distinct ranges of downstream responses. This review focuses on the mechanisms by which the production a common product, PIP3, can produce isoform-specific signaling by PI 3-kinases.

Phosphatidylinositol-3,4,5-trisphosphate (PIP3) is the exclusive product of the Class I PI 3-kinases, which are large heterodimeric proteins consisting of a 110 kDa catalytic subunit (p110 α , p110 β , p110 δ , or p110 γ) bound to a regulatory subunit (p85 α , p85 β , p50 α , p55 α , p55 β , p87 or p101)[1]. Depending on the isoform, the Class I PI 3-kinases are activated in response to ligand stimulation of both receptor tyrosine kinases and G-protein-coupled receptors. Although activated downstream of kinases, these enzymes are not activated by direct phosphorylation. Instead, their activity is regulated by transient binding to tyrosine phosphorylated proteins, G α subunits from trimeric G-proteins, small GTPases in the Rho and Ras family, and SH3 domain-containing proteins.

Despite the fact that all of these enzymes utilize the same substrate, PI[4,5]P2, and produce the same lipid, PIP3, extensive studies in cell culture and in model organisms have demonstrated that the different PI 3-kinase isoforms produce a wide variety of distinct downstream responses. Isoform-specific signaling downstream from Class I PI 3-kinase has been reviewed in depth [2]. This article will instead focus on potential mechanisms by which the production of PIP3 by different PI 3-kinases can lead to divergent signals.

PIP3 works by recruiting downstream effectors containing lipid-binding domains

PIP3 was first identified by Cantley and colleagues in PDGF-stimulated fibroblasts [3]. The lipid was distinguished from other phosphoinositides by the mobility of its deacylated head group (gro-PIP3) on an anion-exchange HPLC column. The lipid can also be identified by its slow mobility, relative to phosphatidylinositol and mono- or bis-phosphoinositides, during thin layer chromatography [4]. More recent methods for the analysis of PIP3 have included displacement assays or blot-based assays with recombinant PIP3 binding proteins [5,6], and mass spectrometric methods [7].

Unlike PI[4,5]P₂, which can be converted from a signaling lipid to the soluble second messengers IP₃ and diacylglycerol by phospholipase C-mediated, 3-phosphoinositides are not substrates for phospholipase C [8]. Instead, they signal by binding to and recruiting effector proteins containing specific lipid binding domains. The first identified PIP₃ effector was the protein kinase Akt/PKB, which contains a Pleckstrin Homology Domain (PH domain) that binds to PIP₃ and PI[3,4]P₂ and is required for Akt activation by PI 3-Additional PIP₃-binding proteins include GRP-1, ARNO and centaurin-1, which are exchange factors for the ARF GTPases, cytohesin which regulates integrin signaling, and the Tec-family tyrosine kinase BTK[9]. The presence of a tandem Dbp-homology domain-PH domain was also identified as a common feature of guanine nucleotide exchange factors (GEFs) for Rho-family GTPases, including PIP₃-regulated GEFs like Tiam1 and Vav [10,11]. In all of these cases, binding to PIP₃ required an intact PH domain. It is important to note that only a subset of PH domains bind PIP₃; others bind to different phosphoinositides, or do not bind lipids at all [9].

These studies led to a model in which production of PIP₃ at the cytosolic face of cellular membranes leads to the recruitment and, in some cases, the allosteric activation of the effector proteins. The mechanism is most fully developed for Akt, where PIP₃ binding to the Akt PH domain drives 3 related events: targeting of Akt to the cell membrane, which is coincident with membrane targeting of the upstream Akt activator PDK-1, followed by a conformational rearrangement of AKT that facilitates PDK-1 mediated phosphorylation on T308 in the Akt activation loop [12]. However, it must be noted that while lipid binding contributes to the membrane recruitment of some PH domain-containing proteins, protein-protein interactions may serve to further refine the site of recruitment, as well as the stability and duration of targeting. For example, the PH domains from Akt, ARNO, Btk and GRP1 all bind to PIP₃, yet their expression in cells caused distinct inhibitory effects on cellular signaling, and mutation of residues not involved in PIP₃ binding abolished these effects [13]. These data suggest that PIP₃-independent interactions affect the location, stability or duration of PIP₃-mediated targeting. In some cases, PIP₃ binding to PH domains can regulate enzyme activity independently of membrane recruitment. For example, in the Arf GAP ARAP1, the PH domain is required for PIP₃-stimulated GAP activity, yet does not bind tightly enough to PIP₃ to mediate membrane binding [14]. In this case, membrane recruitment by a PIP₃-independent mechanism, such as protein-protein interaction, facilitates allosteric regulation of the GAP by PIP₃ binding to the PH domain. Overall, it seems likely that the specificity of PH domain-mediated signaling involves a combination of lipid mediated and protein mediated targeting.

In the repertoire of downstream signaling events initiated by production of PIP₃, activation of the Akt kinase plays an outsized role [15]. The 3 isoforms of this serine/threonine protein kinase have major roles in the regulation of metabolism, and protein and lipid synthesis and degradation, through the FOXO family of transcription factors, the Glycogen Synthase Kinase 3 protein kinase, the AS160 regulator of glucose transport trafficking, and the mTOR protein kinase, among other downstream effectors. Akt also regulates survival pathways, inflammatory signaling, the cell cycle, and cardiovascular homeostasis. Other major effectors of PIP₃ include guanine nucleotide exchange factors involved with vesicular trafficking and cytoskeletal regulation [16], and the Tec tyrosine kinases that regulate signaling in lymphocytes and mast cells [17]. The downstream effectors of PIP₃ have been described in detail in recent reviews [18,19], and will not be discussed further here.

Class I PI 3-kinases are regulated by protein-protein interactions

The Class I PI 3-kinases are subdivided into the Class IA enzymes, in which the regulatory subunits have two SH2 domains that interact with tyrosine-phosphorylated proteins, and the

Class IB enzymes, which interact with a distinct pair of structurally uncharacterized regulatory subunits (Figure 1). The Class IA heterodimer, which consists of an 85, 55 or 50 kDa regulatory subunit bound to one of three 110 kDa catalytic subunits, is extremely stable, and is essentially irreversible once formed [1]. The Class IB PI 3-kinase heterodimer is comprised of the p110 catalytic subunit and either the p101 or p87 regulatory subunits [2]. We know of four types of regulatory interactions that activate heterodimeric PI 3-kinases.

- a. *Disruption of SH2 domain-mediated inhibition.* For the Class IA PI 3-kinases, displacement of inhibitory contacts between the p85 SH2 domains and the p110 catalytic subunit activate the enzyme [20–22]. Inhibitory interactions involving both the n-terminal and c-terminal SH2 domains have been demonstrated [23], although there is controversy as to whether the cSH2 domain is inhibitory for p110 [24]. The consensus is that SH2 domain binding to activated receptor tyrosine kinases or their substrates disrupt the inhibitory contacts and activates the p85/p110 dimer.
- b. *Membrane targeting.* Class I PI 3-kinases interact with RTKs, activated Ras, activated Rac, G subunits, and SH3 domains from Src-family tyrosine kinases [1]. All of these proteins are integral membrane proteins or are membrane associated, through the covalent attachment of myristoyl, palmitoyl and isoprenoid lipids. Given that the substrate of the PI 3-kinases is a membrane lipid, increased membrane targeting is sufficient to increase the rate of PIP3 production by PI 3-kinases. This was most clearly shown by the hyperactive phenotype induced by direct membrane targeting of PI 3-kinase catalytic subunits by addition of a C-terminal CAAX motif [25]. Using deuterium exchange-mass spectrometry methods, Williams and coworkers have noted that the binding of tyrosine phosphorylated peptides to p85 SH2 domains leads to conformational changes that enhance p110 and p110 interactions with membranes [26]. Both p110 and p110 bind to G subunits, which are released downstream from activated G-protein-coupled receptors (GPCRs). For p110, the binding site has been localized to the C2 domain-helical domain linker [27]. Activation by G binding is synergistic with activation of phosphotyrosine peptide binding to the p85 N-terminal SH2 domain, and may act in part by disrupting the inhibitory contact formed by this domain. However, activation by G is also seen with monomeric p110 or p110 [28], suggesting that a large component of G-mediated activation is due to membrane targeting. A similar mechanism has been suggested for the activation of p101/p110 by G [29].
- c. *Allosteric activation by small GTPases binding.* As mentioned above, both small GTPases and trimeric G-proteins directly interact with Class I PI 3-kinase. All the p110 catalytic subunits contain a Ras-binding domain, although direct binding to Ras-GTP has only been demonstrated for p110, p110 and p110 [30]. A crystal structure of the p110/Ras-GTP complex indicated conformational changes in the catalytic cleft, which presumably increases enzyme activity synergistically with membrane targeting [31]. Interestingly, it has been shown that activation by Ras preferentially occurs with the p87/p110 dimer, as opposed to with the p101/p110 dimer [32], suggesting that the Class IB regulatory subunits influence the interactions of p110 with Ras. Finally, binding of activated Rac or Cdc42 to the so-called BCR homology domain in the p85 subunit activates p85/p110 dimers in vitro [33], all this has not been demonstrated in intact cells. The mechanism of activation is not known, as there is little information on the conformation/structure of the N-terminal half of p85.
- d. *Disruption of SH3/proline rich domain binding.* The SH3 domain of p85, which resides at the extreme N-terminus of the protein, has been shown to bind to

peptides derived from the internal proline-rich domains (PRDs) in p85. These data suggest that the N-terminus of p85 may exist in a closed conformation mediated by intramolecular SH3-PRD interactions. Binding events that would be predicted to disrupt such a conformation activate p85/p110 dimers. For example, p85/p110 dimers are activated by SH3 domains Src-family kinases [34], which can bind to p85 PRDs. While these data suggest a model in which exogenous proline-rich peptides or SH3 domains disrupt an inhibitory intramolecular SH3-PRD interaction, there is no direct evidence supporting this model. Given that the BCR domain lies between the two proline-rich domains, it is possible that Rac/Cdc42 binding to the BCR domain also functions by disrupting intramolecular SH3-proline-rich domain interactions.

Activating oncogenic mutations are commonly found in the p110 catalytic subunit [35], as well as in the p85 regulatory subunit [36,37]. The mechanisms by which these mutations activate PI 3-kinase are in some cases known. For example, the frequently mutated E545K residue in the p110 helical domain disrupts the inhibitory contact made by the N-terminal SH2 domain [20], and thus mimics phosphotyrosine-mediated activation. In contrast, a second commonly mutated site in p110, H1047R, acts by increasing the basal association of p85/p110^{aga}; dimers with membranes [21]. The net effect of most activating mutants of p110 appears to be to induce conformational changes that increase membrane binding [26]. Mutants of both p85 and p110 in human tumors have been described; these mutants occur at an inhibitory contact between the C2 domain of p110 and the iSH2 domain of p85 [36,38,39]. It appears that these mutations mimic a loosening of the C2 domain-iSH2 domain interface that occurs in response to phosphotyrosine-mediated displacement of the inhibitory nSH2 domain [26].

PIP3 phosphatases shape the kinetics of PIP3 signaling

The importance of the timely elimination of PIP3-mediated signals, once they are initiated, is illustrated by the fact that the PIP3 3-phosphatase, Phosphatase and Tensin Homology (PTEN), is frequently mutated or eliminated in human cancers [40,41]. The normal functioning of metazoan cells requires that the PIP3 signal be sharply constrained in both time and space.

The half-life of PIP3 in cells has been difficult to measure, but appears to be quite short. Using a PIP3 antibody to measure the decay in EGF-stimulated plasma membrane PIP3 induced by acute treatment with the PI 3-kinase inhibitor LY294002, the half-life for PIP3 was estimated to be less than 5 sec [42]. Of note, this study also found that a commonly used method to measure PIP3 levels in live cells, expression of GFP-labeled PH domains, led to an approximately 5-fold increase in the half-life of PIP3. This effect was presumably due to the sequestration of PIP3 by PH domain overexpression. This analysis suggests that measurements of PIP3 dynamics using PH domain probes, which have estimated the half-life of PIP3 to be approximately 1 min [43], likely overestimate the duration of the PIP3 signaling.

The overall dynamics of the cellular PIP3 response is stimulation dependent. In fMLP-stimulated neutrophils, PIP3 levels peaked by 20 sec and declined to close to basal levels by 2 min [44]. In PDGF-stimulated fibroblasts, PIP3 peaked by 40 sec but remained elevated at 5 min [45]. Other studies have suggested that sustained elevations of PIP3 occurs in response to acute stimulation of receptor tyrosine kinases [3,46]. The lifetime of the PIP3 signal in specialized cases is more transient. For example, in macrophages, there is an accumulation of PIP3 in the phagocytic cup during FcR γ -mediated phagocytosis; PIP3 disappears during closure of the phagosome, over a period of 2–3 min [47,48]. In *Dictyostelium* acutely exposed to a chemoattractant gradient, PIP3 accumulates at the

leading edge in a biphasic manner, with a transient peak at 20 sec followed by a sustained elevation at 2 min[49].

The rapid turnover of newly synthesized PIP3 occurs either through removal of the 3-phosphate by PTEN, returning the lipid to the PI[4,5]P2 precursor, or through removal of the 5-phosphate by phosphatases such as SHIP or synaptojanin [50]. PTEN-mediated hydrolysis clearly terminates the PIP3 signal, and numerous studies have shown that inactivation or deletion of PTEN leads to increased levels of PIP3 [40]. The downstream effects of the activity of the 5-phosphatases is less clear, since their product is a lipid that can still signal via PH domains that bind to PI(3,4)P2, including those of Akt or Tapp1 [51,52]. Nonetheless, knockout of SHIP enhances the inflammatory response of myeloid and mast cells [53,54], clearly indicating its role as a negative regulators of PI 3-kinase signaling.

Interestingly, studies in Dictyostelium have shown that PTEN may contribute to certain PIP3-mediated cellular responses, particularly those that require an asymmetric spatial distribution of the lipid. cAMP-stimulation of Dictyostelium leads to an acute and spatially restricted accumulation of PIP3 at the leading edge of the cell [55]. This is accomplished at least in part by PTEN localization to the sides and rear of the cell, and its exclusion from the leading edge [56]. The positive role of PTEN in the chemotactic response is demonstrated by the impaired directionality of PTEN null cells, which produce poorly coordinated protrusions that reduce movement toward the chemoattractant source [57].

The biology of PTEN deletion involves phenotypes that are more complex than can be explained simply by an increase in PIP3 levels or lifetime. PTEN has a protein phosphatase activity whose disruption leads to distinct phenotypes [58,59]. PTEN also has signaling functions in the nucleus that do not appear to involve dephosphorylation of PIP3 [41,60].

How do different Class I PI 3-kinase isoforms achieve signaling specificity?

The distinct functions of the Class I PI 3-kinases have been studied in vitro, in cells and in animal models. Abundant evidence has accumulated showing that these lipid kinases perform quite different functions [2]. While isoform specific signaling by a protein kinase could easily be due to distinct substrate specificities, all the Class I PI 3-kinases make the same product, PIP3. In that case, how is isoform-specific signaling achieved? The question of how different p110 isoforms perform distinct tasks is especially interesting for the Class IA catalytic subunits, which share the same regulatory subunits and therefore should have similar interactions with tyrosine phosphorylated proteins.

Selective expression

To some extent, the dominant PI 3-kinase in a given response depends on which PI 3-kinase is expressed in the cell. While both p110 α and p110 β are ubiquitously expressed, the ratios of these two catalytic subunits may vary considerable. For example, mass spectroscopic analysis shows that p110 α predominates in NIH3T3 cells, and in fat, liver and brain, whereas p110 β and p110 δ are expressed at similar levels in muscle [61]. Similarly, the predominant expression of p110 α and p110 β in hematopoietic cells explains the anti-inflammatory phenotypes observed in kinase dead knock-in mice for these p110 isoforms [62,63]. Interestingly, these patterns of expression are disrupted in cancer. For example, increased expression of p110 α and p110 β have been observed in many non-hematopoietic cancers [2].

However, the expression pattern for the p110 isoforms is often insufficient to explain which isoform mediates a given cellular response. For example, 3T3 L1 adipocytes express 3–4 fold more p110 α than p110 β whereas L6 myotubes express similar levels of both isoforms.

Selective inhibition of p110 decreased PIP3 production in response to insulin in both cell types, whereas an inhibitor of p110 had only a partial effect in 3T3L1 cells and no effect in L6 cells [64]. Similarly, rat adenocarcinoma cells express similar levels of p110 and p110, yet p110 is selectively involved in EGF-stimulated protrusive responses [65].

One complication of this type of comparison is that the published data on the enzyme kinetic properties of the different Class I PI 3-kinases under different activation conditions is sparse. Using PIP2 as substrate, the maximal in vitro activity of purified p85/p110 from insect cells was 5-fold higher than that of p85/p110 or p85/p110 or monomeric p110; dimers of p110 with p101 or p87 were not tested [66]. Similarly, Shepherd and colleagues found that p85/p110, expressed in mammalian cells, was 4-fold more active than p85/p110 using PI as a substrate, although activities were similar using PIP2. This study also found that the activity ratio of p110 versus p110 was reversed at low substrate concentrations, suggesting a lower K_m for lipid for p110 [67]. In contrast, a comparison of the Class IA PI 3-kinases using a scintillation proximity assay found that the K_m for ATP was 26–28 μM for all three p85/p110 dimers; the ATP K_m increased to 43 μM p110/p55 [68]. All of these measurements were made under basal conditions; a careful comparison of the substrate K_m and the maximal specific activity of each isoform in the presence of single and multiple activators (e.g. RTK plus Ras) is still lacking.

Mechanisms of activation: RTK/G-protein synergy

As discussed above, the three Class IA PI 3-kinase exhibit differences in their responsiveness to G-proteins, which could explain some of their distinct signaling outputs. For example, p110 and p110 both interact directly with activated Ras in a manner that could synergize with activation via SH2 domain occupancy. In contrast, p110 has not been shown to be activated by Ras, but is activated synergistically by SH2 domain occupancy and direct binding to G subunits from trimeric G-proteins [28]. p110 is activated by both Ras and G subunits [69], although it has been suggested that these are independent events mediated by the p101 versus p87 regulatory subunits [32]. Recent studies have also detected activation of p110 downstream from selected receptor tyrosine kinase and Toll-like receptors [70]. Taken together, these data suggest that in response to a common ligand (e.g. insulin), the coordination of the insulin receptor/p-IRS-1 signal with concurrent signals from GPCRs or Ras could lead to distinct amplitudes or durations of signaling output (production of PIP3) from different PI 3-kinases, as well as distinct locations of PIP3 accumulation (see below).

Targeting of PI 3-kinases: plasma membrane, rafts and endomembranes, and the nucleus

The production of PIP3 at specific locations via PI3-kinase targeting is not well understood. A major stumbling block has been the absence of good antibodies for the detection of the endogenous enzymes. Overexpression studies on epitope- or fluorescent protein-tagged versions of the Class IA catalytic subunits are particularly difficult to interpret, since the overexpressed enzymes compete with endogenous enzymes for p85 adapter subunits. For example, in rat hepatoma cells that require p110 for EGF-stimulated cytoskeletal responses [65], overexpression of wild type p110 beta acts as a dominant negative [71], presumably by competing for a limiting pool of p85. It is hoped that these issues will be addressed in the future by arduous but definitive methods such as knocking in tagged PI 3-kinases into somatic cells or mice.

Despite these deficiencies in our knowledge, there is good evidence for the accumulation of PIP3 in a number of distinct subcellular locations. Most clear is the plasma membrane, where GFP-PH domain probes, anti-PIP3 immunostaining and electron microscopy using gold-labeled PH domains have shown the rapid accumulation of PIP3 in response to both

RTK and GPCR ligands [42,43,60]. These data are consistent with the localization of the major allosteric activators of Class I PI 3-kinases: receptor tyrosine kinases like the PDGF and CSF-1 receptors, lipidated GTPases like Ras and Rac, and lipidated G subunits from trimeric G-proteins. Since the PI 3-kinases are largely cytosolic under basal conditions, the localized activation of distinct PI 3-kinase depends on the localization of their allosteric activators.

Within the plasma membrane, differential signaling by PI 3-kinase could occur via the partitioning of PI 3-kinase activators into and out of lipid rafts, which are regions characterized by relatively high levels of cholesterol, sphingolipids, glycosphosphatidylinositol-anchored proteins and glycolipids [72]. Studies by Hope and Pike demonstrated enrichment of the Class I PI 3-kinase substrate PIP2 in lipid rafts [73], although this concept has been controversial [74]. Moreover, studies on the localization of fluorescent PH domains and FRET-based biosensors have documented the appearance of PIP3 in lipid rafts [75–77]. Gao and Zhang measured the production of PIP3 and activation of Akt using fluorescent biosensors that could be specifically targeted to raft versus non-raft domains of the plasma membrane [78]. PI 3-kinase activation by insulin was highly raft-dependent, whereas responses to PDGF were only partially diminished by pharmacological disruption of rafts. These data suggested that insulin stimulated PI 3-kinase activation occurred primarily in rafts. In contrast, Lassere et al have suggested that the concentration of PIP3 in rafts is induced by PIP3 binding proteins, and does not imply that the PIP3 is actually synthesized in rafts [79].

How might rafts achieve isoform specific activation of PI 3-kinase? Some receptor tyrosine kinases have been reported to be concentrated in rafts [80–82], and if these receptors preferentially bound a subset of Class IA isoforms, this could achieve specific signaling. Unfortunately, since all the Class IA isoforms share the same p85 subunits, it is not clear how this would generate specificity. Although numerous reports have shown that G subunits and G subunits from trimeric G-proteins are enriched in rafts [83–86], isolated G subunits may actually be excluded from rafts [81]. Thus, it is not clear whether G interactions with PI 3-kinases would occur within rafts, or in non-raft regions after G dissociation from G. Furthermore, as far as we know, p110 and p110 are activated by similar isoforms of G subunits [87], so this targeting would not lead to differential signaling between the two isoforms. The palmitoylated isoforms of Ras, like N-Ras and H-Ras, are raft associated, with GTP-loaded N-Ras showing increased partitioning into rafts [88]. In contrast, K-Ras is largely excluded from rafts [89]. If, for example, N-Ras preferentially interacts with p110, p110 or p110, then rafts could mediate isoform-specific activation. Although all Ras isoforms (N-, H- and K-Ras as well as R-Ras) bind to p110, p110 and p110 in vitro (Julian Downward, personal communication), the net activation of PI 3-kinase signaling by distinct combinations of Ras and PI 3-kinase has not been systematically evaluated in vivo. One study did evaluate the effects of distinct Ras isoforms on the four p110 catalytic subunits in cells; p110, p110 and p110 were activated by Ras, but only p110 showed selectivity (for the Ras homologs R-Ras and TC21) [90]. However, since the catalytic subunits were expressed without their regulatory subunits, the findings are difficult to interpret.

In contrast to the clear evidence that the bulk of PIP3 production occurs in the plasma membrane, evidence for the production of PIP3 in endomembranes is more limited. The EM study of Lucocq and colleagues estimated that no more than 5% of total PIP3 was in any endomembrane compartment [60], but physiologically important signals could be generated from local concentrations of this signaling molecule. Several studies have shown that the PI 3-kinase substrate PI(4,5)P2 is present in endosomes [91,92]. Using a PIP3 biosensor that was directed to endomembranes via a palmitoylation-deficient Ras CAAX motif, PDGF-

stimulated PIP3 production in endomembranes was observed [93]. The signal was abolished by mutant dynamin, suggesting that it was caused by the endocytosis of activated PDGF receptors. Similarly, Wang et al. used a combination of monensin and a PDGFR inhibitor to drive the accumulation of ligand-bound but inactive receptors in endosomes. Upon removal of the inhibitors, activation of the endosomal PDGF was sufficient to activate downstream effectors [82]. This suggests that endosomal PI 3-kinase signaling is feasible. Studies on the PIP3-stimulated kinase Akt in fact have suggested endosomes as an important compartment for Akt signaling, particularly in conjunction with the Rab5-associated endosomal protein APPL [94]. However, given that Akt activation continues after its dissociation from PIP3, it is possible that Akt is recruited from a plasma membrane site of activation, rather than being activated in situ at the endosome by PIP3.

In an interesting variation on the story of endosomal Class I PI 3-kinases, Zerial and colleagues demonstrated the presence of the p110 catalytic subunit in endosomes [95], and suggested that its colocalization with lipid phosphatases was an important source of endosomal PI[3]P rather than PIP3 [96]. However, the endosomal functions of p110 appear to be independent of its lipid kinase activity [97,98], so the presence of p110 in endosomes does not necessarily imply the presence of PIP3. In contrast, in degranulating mast cells, p101/p110 was shown to produce a rapidly endocytosed pool of PIP3 that was not observed in cells expressing p87/p110 [99]. In this case, endosomal PIP3 is derived from the plasma membrane, so the presence of endosomal PIP3 does not imply the presence of a class I PI 3-kinase.

How might distinct PI 3-kinases be differentially targeted to endosomes? The co-internalization of PDGF receptors and p85 was visualized in an early paper by Corvera and Cantley [100], but this study did not distinguish between p85-bound p110 isoforms. Similarly, activation of PI 3-kinase in insulin-stimulated cells is mediated by binding to the IRS-1 scaffold protein [101], which is localized to poorly defined intracellular membranes [102,103]. Although Vanhaesebroeck and colleagues suggested that IRS-1/2 preferentially recruits p85/p110 in insulin-stimulated liver, muscle and fat [104], the mechanism for this selectivity is not clear. As noted above, p85/p110 dimers directly interact with the early endosomal GTPase Rab5 [95], providing a unique localization signal for this isoform. We have recently defined residues in the helical domain of p110, Q596 and I597, whose mutation abolishes p110 binding to Rab5 (R.S. Salamon, H.A. Dbouk and J.M. Backer, unpublished observations). Studies on the phenotype of cells expressing these mutants will directly evaluate the role of endosome-specific signaling from p110.

Finally, Lucocq and Downes reported a PDGF-stimulated pool of nuclear PIP3 [60]. Studies have shown that both p110 and p110 are present in the nucleus [105,106], and the CSF-1 receptor has been shown to traffic to the nuclear envelope, where it activates Akt in a p110-dependent manner [107]. Nuclear localization would therefore not distinguish signaling by p110 and p110, but might distinguish signaling by p110 versus p110.

Isoform specific signaling by PI 3-kinases requires combinatorial inputs

Taken together, it does not seem as if any one activation or targeting mechanism can produce a specific or preferential activation of any given PI 3-kinase isoforms (Figure 2). In some cases, the predominance of one isoform over another may be explained by differences in the kinetic properties of the isoforms; this seems most likely to be the case for p110, given its higher basal activity [66,67]. Alternatively, isoform-specific PI 3-kinase signaling, in a cell that contains multiple isoforms, may require a combination of localization constraints and synergistic activation by multiple activators (Table 1). For example, in a growth factor-stimulated cell, p85/p110 and p85/p110 might both reside in an endosome,

via receptor tyrosine kinase binding for the former and Rab5 binding for the latter. If the growth factor were a potent activator of endosomal Ras, this would synergistically activate p85/p110 but not p85/p110 β , and lead to a signal that was primarily p110 β -dependent. A combined RTK/GPCR signal might preferentially activate p85/p110 β , whereas a combined GPCR/Ras signal might preferentially activate p87/p110 β . Of all the isoforms, differential signaling by p110 α and p110 β was the first to be described [108] and remains the hardest to understand. Both isoforms bind RTKs and Ras, and both undergo autoinhibitory autophosphorylation (although by different mechanisms [109,110]). The ability of RTKs to distinguish between these isoforms may reside in the still mysterious cellular code for the matching of p110 isoforms (p110 α , p110 β , and p110 δ) with p85 isoforms (p85 α , p55 α , p50 α , p85 β and p55 β).

Finally, it should be noted that PI 3-kinases have activities not directly related to PIP3 production, which may affect signaling output. All of the Class I PI 3-kinases have protein kinase activity. Although in most cases the activity seems to be limited to autophosphorylation [109–111], a protein kinase-only mutant of p110 α was able to signal to Erk [112] and a limited number of the protein substrates have been described [113,114]. In addition, PI 3-kinases have been shown to have biological activities that do not require an active kinase domain [115]. For example, mice expressing kinase dead p110 α are viable, although infertile, whereas homozygous knockout of p110 α produces embryonic lethality [97,98,116]. p110 β , in particular, has been shown to regulate contractility in the heart by serving as a scaffold for phosphodiesterase 3B and G-protein-coupled receptors kinase 2, which regulates the β_2 -adrenergic receptor [117]. The scaffolding activity of the PI 3-kinase regulatory and catalytic subunits is likely to contribute to the spectrum of downstream responses to distinct PI 3-kinase isoforms.

Conclusions and outlook

The focus of much of current research on PIP3-mediated signaling is on the identification of novel downstream effectors, but there is still a great deal to be learned about the activation, targeting and specificity of the enzymes producing and regulating the production of PIP3 itself. A review of our knowledge of the activation mechanisms for Class I PI 3-kinases suggests that differential outputs from the distinct PI 3-kinase isoforms depends on different combinations of simultaneous upstream inputs, different sites of action, as well as contributions from kinase-independent scaffolding functions. Given the compelling data linking aberrant regulation of Class I PI 3-kinase to pathological outcomes, a more precise understanding of how these enzymes are regulated in cells should hold considerable promise for novel interventions into human disease.

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List of Abbreviations

ARAP1	Arf GAP with Rho GAP domain, ankyrin repeat, and PH domain 1
Arf	ADP ribosylation factor
ARNO	ADP-ribosylation factor (ARF) nucleotide-binding site opener
BTK	Bruton's tyrosine kinase

GAP	GTPase-Activating Proteins
GEF	Guanine nucleotide exchange factors
GRP-1	General receptor for 3-phosphoinositides 1
PDGF	Platelet-derived growth factor
PDK-1	Phosphoinositide dependent kinase-1
PI(3,4)P₂	phosphatidylinositol (3,4) bisphosphate
PI(4,5)P₂	phosphatidylinositol (4,5) bisphosphate
PIP₃	phosphatidylinositol (3,4,5) trisphosphate

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Box 1**PIP3 – Unexplored areas**

Although PIP3 production and destruction is a critical component of PI 3-kinase signaling, it is not clear that all PIP3 molecules are the same. Mass spectrometry studies on the acyl chain distribution of PIP3 have been inconsistent. A study in RAW 264.7 and primary murine macrophages showed significant differences in the fatty acyl composition of PIP3 molecules produced in response to ligands for distinct receptor types (LPA, C5A, Zymosan and MCF) [118]. In contrast, Clark et al. found that the production of C18:0/C20:4 (stearoyl/arachidonyl) PIP3 predominated in fMLP-stimulated neutrophils, EGF-stimulated MCF10A breast cancer cells, and insulin stimulated fat and liver [7]. A striking finding in the latter study was that the production of C18:0/C20:4 PIP3 was greatly in excess of its proportion in the overall PIP2 pool, suggesting a significant role of the fatty acid chains in the ability of PI 3-kinase to utilize substrate. It will be interesting to test whether this preference is altered under conditions in which one or more PI 3-kinase isoform is silenced or inhibited.

A second unanswered question with regard to PIP3 is whether it stays where it is put, i.e. whether the lipid can move laterally within membranes. While the half-life of bulk PIP3 in a cell is short, its migration into membrane subdomains such as rafts could lead to prolonged lifetimes for local PIP3 pools. Finally, it is interesting to note a recent study suggesting that increases in intracellular divalent cations, including Ca^{+2} and Mg^{+2} , can induce the clustering of PIP2 in model membranes [119]. The authors suggest that cation-induced clustering of PIP2 could affect the ability of PIP2-binding proteins to bind PIP2 at the plasma membrane. While the study did not examine PIP3, it seems possible that a similar mechanism might cause PIP3 clustering, locally increasing PIP3 concentrations and modulating interactions with PIP3 binding proteins.

Figure 1. Domain Organization and Activators of Class I PI 3-kinases

The Class IA PI 3-kinases consist of a p85 regulatory subunit containing SH3, BCR, proline-rich, SH2 and coiled-coil (iSH2) domains, and a p110 catalytic subunit (in yellow) containing an Adapter Binding Domain (ABD), a Ras Binding Domain (RBD), C2, helical and kinase domains. Shorter isoforms of p85 (p55, p50, p55) contain a truncated N-terminal segment linked to the first SH2 domain. The Class IB PI 3-kinase consists of structurally uncharacterized p87/84 or p101 regulatory subunits bound to a p110 catalytic subunit (in yellow), whose domain organization is similar to that of Class IA PI 3-kinases. The mechanism of p87/p101 binding to p110 is not yet known, but involves both the N- and C-terminal regions of p110 [120]. Of the upstream activators shown, Ras binding regulates p85/p110 and p85/p110 but not p85/p110. G regulates p85/p110 and p101/p110 but not p85/p110 or p85/p110.

Figure 2. Pathways to isoform-specific PI 3-kinase activation

While no one PI 3-kinase activator yields specific or preferential activation of any one PI 3-kinase isoform, combinations of activators and distinct activator locations could allow differential activation of distinct PI 3-kinases.

Table 1

Activators of the Class I PI 3-kinases.

	RTKs- pY	G	Ras
p85/p110	X		X
p85/p110	X	X	
p85/p110	X		X
P87/p110		X	X

Synergistic activation by RTKs plus GPCRS (for p85/p110) and GPCRs plus Ras (for p87/p110) could produce specific activation.