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Signaling by Distinct Classes of Phosphoinositide 3-Kinases

B. Vanhaesebroeck*,[1] and M. D. Waterfield*,†

*Ludwig Institute for Cancer Research, 91 Riding House Street, London W1P 8BT, United Kingdom; and †Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, United Kingdom

Many signaling pathways converge on and regulate phosphoinositide 3-kinase (PI3K) enzymes whose inositol lipid products are key mediators of intracellular signaling. Different PI3K isoforms generate specific lipids that bind to FYVE and pleckstrin homology (PH) domains in a variety of proteins, affecting their localization, conformation, and activities. Here we review the activation mechanisms of the different types of PI3Ks and their downstream actions, with focus on the PI3Ks that are acutely triggered by extracellular stimulation. © 1999 Academic Press

INOSITOL PHOSPHOLIPIDS

Inositol-containing lipids are a class of phospholipids, consisting of phosphatidic acid to which an inositol ring is attached via its 1′-OH group (Fig. 1). If this inositol ring carries no phosphates this lipid is called phosphatidylinositol (PtdIns).

In cells, all free -OH groups of the inositol ring of PtdIns—apart from those at the 2′ and 6′ positions—can be phosphorylated, in different combinations. A phosphorylated derivative of PtdIns is called a phosphoinositide (PI). It is the constellation of the phosphate groups around the inositol head group that provides each PI with unique functional roles in cells.

PI3K LIPID PRODUCTS

PI3Ks phosphorylate the 3′-OH position of the inositol ring of PtdIns and some specific PIs. The 3′-PI species found in mammalian cells are PtdIns(3)P, PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PtdIns(3,4,5)P_3 (with the latter lipid frequently being referred to as PIP3).

The basal levels of PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PIP3 in cells are very low and can rise sharply upon cellular stimulation (see below). This discriminates these lipids from the other PIs whose basal levels are substantial and either weakly increase [e.g., PtdIns(3)P], remain more or less constant [e.g., PtdIns(4)P], or decrease [as in the case of PtdIns(4,5)P_2] upon cleavage by phospholipase C (PLC; Fig. 1).

PI3K lipid products are not substrates for the PI-specific PLC enzymes that cleave inositol phospholipids into membrane-bound diacylglycerol and soluble inositol phosphates (Fig. 1). This separates PI3K signal transduction from the "classical" PLC/PtdIns(4,5)P_2 pathway that leads to among others Ca^{2+} release [1, 2]. Instead, 3′-PIs are converted by kinases and phosphatases that act on the inositol ring [3–5]. An example is the 5′-phosphorylation of PtdIns(3)P which gives rise to the recently discovered PtdIns(3,5)P_2 [6, 7]. Likewise, 5′-phosphatase action on PIP3 is most likely responsible for a large fraction of the cellular PtdIns(3,4)P_2 [8]. A 3′-phosphatase that has received a lot of attention recently is PTEN, a previously known tumor suppressor gene that converts PtdIns(3,4)P_2 to PtdIns(4)P and PIP3 to PtdIns(4,5)P_2. The gene encoding this protein is mutated and inactivated in a significant number of human cancers. This results in elevated concentrations of PtdIns(3,4)P_2/PIP3 leading to constitutive PI3K signaling in those cells [9, 175].

DIFFERENT CLASSES OF PI3Ks RESPOND TO DIFFERENT EXTRACELLULAR STIMULI AND GENERATE SPECIFIC 3′-PIs IN CELLS

There are multiple isoforms of PI3Ks that can be divided in three classes [10–12]. All PI3K catalytic subunits share a homologous region that consists of a catalytic core domain linked to the so-called PIK (PI kinase homology) domain with unknown function (Fig. 2).

Class I PI3Ks

Class I PI3Ks are heterodimers made up of a ~110-kDa catalytic subunit (p110) and an adaptor/regulatory subunit.

In vitro, these PI3Ks can utilize PtdIns, PdIns(4)P, and PtdIns(4,5)P_2 as substrates (Fig. 2). In cells, however, their preferred substrate appears to be PtdIns(4,5)P_2. The resulting PIP3 is then believed to

[1] To whom correspondence and reprint requests should be addressed: Fax: +44 [0]20-7878.4040. E-mail: bartvanh@ludwig.ucl.ac.uk.

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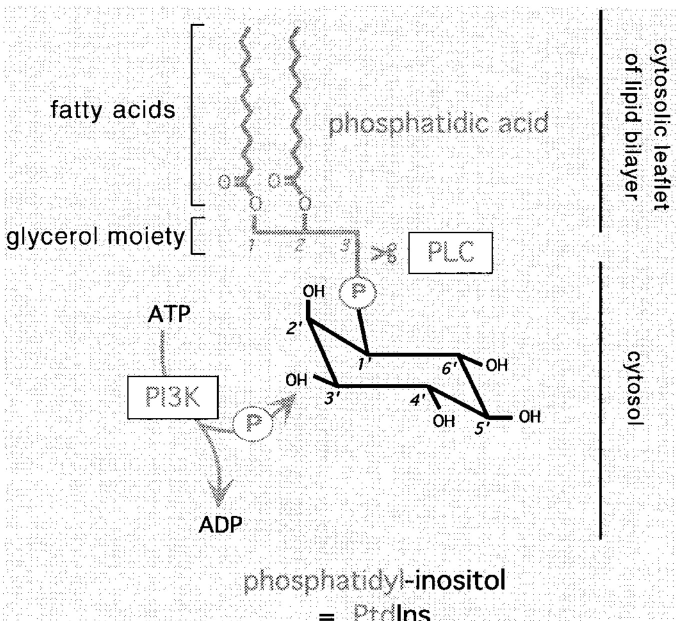


FIG. 1. Simplified representation of phosphatic acid and PtdIns and the points of action of PLC and PI3K. For further details and an overview of the structure/nomenclature of the inositol ring, see Refs. [1, 6, 173].

Phosphatidic acid consists of a glycerol backbone in which all of the three hydroxyl groups are bound (esterified) to acids (fatty acids at positions 1 and 2 and phosphoric acid at position 3).

Phosphatidyl-X (Ptd-X), derivative of phosphatidic acid with a chemical component X bound to the phosphate group. Examples of X in cells are inositol (Ins), serine, choline, and ethanolamine. The derivative most relevant for this review is phosphatidylinositol (PtdIns). By convention, the numbers indicating the carbons in X carry a prime, in contrast to the carbons of the glycerol backbone.

Phosphoinositide (PI), PtdIns whose inositol head group carries one or more additional phosphate groups.

Phosphoinositide kinase (PIK), enzyme that phosphorylates PtdIns and its derivatives. The term phosphatidylinositol kinase specifies an enzyme that phosphorylates PtdIns only. The name phosphoinositide kinase indicates a broader substrate specificity than PtdIns.

give rise—via the action of 5′-inositol phosphatases—to the typically delayed generation of PtdIns(3,4)P_2 [4, 8]. Class I PI3Ks are further subdivided into class I_A and I_B enzymes, which signal downstream of tyrosine (Tyr) kinases and heterotrimeric G-protein-coupled receptors, respectively. All Class I PI3K members also bind to the monomeric G-protein Ras but the role of this interaction in PI3K signaling is not entirely clear at the moment.

Class I_A PI3Ks

The p110 subunit in these PI3Ks exists in complex with an adaptor protein that has two Src-homology-2 (SH2) domains (Fig. 3). The latter bind to phosphorylated Tyr residues (in a specific context of surrounding amino acids) that are generated by activated Tyr kinases in receptors and various adaptor proteins. This is believed to allow translocation of the cytosolic PI3Ks to the membranes where their lipid substrates and Ras reside (Fig. 3).

Mammals have three p110 isoforms (p110α, β, and δ; encoded by three separate genes) and at least seven adaptor proteins (generated by expression and alternative splicing of three different genes: p85α, p85β, and p55γ). p110α and β are widely distributed in mammalian tissues, in contrast to p110δ which shows a more restricted distribution and is mainly found in leukocytes. A single type class I_A catalytic/adaptor heterodimer is present in *Drosophila melanogaster* (Dp110/p60) and *C. elegans* (AGE-1/AAP-1). The slime mold (*D. discoideum*) has three PI3K catalytic subunits (PI3K1–3) with homology to class I_A PI3Ks. No class I_A PI3K family members have been found in yeast or plants, which is consistent with the absence of PtdIns(3,4)P_2 and PIP3 in these phyla.

All mammalian cell types investigated express at least one class I_A PI3K isoform, and stimulation of almost every receptor that induces Tyr kinase activity also leads to class I_A PI3K activation (for an overview of those stimuli, see [8, 10, 13]). This Tyr kinase activity can be mediated by receptors with Tyr kinase activity (Fig. 3) or by nonreceptor Tyr kinases (such as src-family kinases or JAK kinases) that have been implicated in the activation of class I_A PI3Ks by B- and T-cell antigen receptors, many cytokine receptors, and several costimulatory molecules (such as CD28, CD2, and CD19) as well as in cell–cell and cell–matrix adhesion [14, 15]. Non-Tyr-based PI3K recruitment mechanisms may also contribute to PI3K activation. An example is CD2 which shows a constitutive association with class I_A PI3Ks [16].

Class I_B PI3K

The only class I_B PI3K identified to date is the p110γ catalytic subunit complexed with a 101-kDa regulatory protein (p101) which has no sequence homology to any other known protein (Fig. 3). p110γ/p101 heterodimers are activated by the G$\beta\gamma$ subunits of heterotrimeric G-proteins, and p101 is indispensable for this G$\beta\gamma$ responsiveness [17]. Some groups have reported that class I_B PI3K is responsive to activation by Gα subunits [19, 20].

Class I_B PI3K appears to be present only in mammals where it shows a restricted tissue distribution, being only abundant in white blood cells. This may explain why receptors coupled to heterotrimeric G-proteins do not induce PI3K activity in all cell types. Even in leukocytes that have p110γ, PI3K activity is not always induced upon triggering G-protein-coupled receptors. Therefore, the generation of PtdIns(3,4)P_2/PIP3 via class I_B PI3K is a much less widespread

Mammalian PI3Ks

Class	Structural features of catalytic subunits	Subunits: Catalytic	Subunits: Adaptor	Regulation	Lipid substrates: in vitro	Lipid substrates: in vivo
I A		p110α, β, δ	p85α, p85β, p55γ	Tyr Kinases & Ras	PtdIns, PtdIns(4)P, PtdIns(4,5)P2	PtdIns(4,5)P2
I B		p110γ	p101	heterotrimeric G proteins & Ras		
II		PI3K-C2α, β, δ	?	Tyr kinases? heterotrimeric G proteins? *(not Ras)*	PtdIns, PtdIns(4)P, [PtdIns(4,5)P2]	?
III		Vps34p analogues	p150	Constitutive?	PtdIns	PtdIns

adaptor-binding; Ras-binding; C2; PIK; kinase core domain

FIG. 2. Classes of PI3Ks and the defining features of their catalytic subunits.

mechanism than the synthesis of these lipids through class I_A PI3Ks.

Do Gβγ Subunits Also Activate Class I_A PI3Ks?

Several laboratories have reported that ligands that activate heterotrimeric G-proteins stimulate class I_A PI3K activity [21–25]. This could be explained by Tyr kinase activity known to be induced by stimulation of heterotrimeric G-proteins [26–30]. On the other hand, it appears that p110β but not p110α or p110δ can be directly activated by Gβγ subunits *in vitro* [31, 32]. Consistent with this, neutralizing antibodies to p110β but not p110α block the responsiveness of cells to lysophosphatidic acid, a ligand that signals via heterotrimeric G-proteins ([33] see also Moolenaar, this issue]. Likewise, Gβγ-responsive PI3K activity is found in cytosol from cell lines that do not express detectable levels of p110γ [18]. Further work is needed to establish the *in vivo* contribution of class I_A PI3Ks in PI3K activation downstream of heterotrimeric G-proteins.

Class II PI3Ks

Class II PI3Ks are large molecules (>170 kDa) whose defining feature is a C-terminal C2 domain (Fig. 2). This C2 domain can bind *in vitro* to phospholipids in a Ca^{2+}-independent manner [34, 35]. This Ca^{2+} independence is consistent with the fact that the C2 domain of class II PI3Ks lacks the critical aspartate residues that have been shown in Ca^{2+}-dependent C2 domains to be essential for Ca^{2+} coordination (discussed in Ref. [34]). The large N-terminal regions of class II PI3Ks show no homology to any known protein, and there are no indications for binding of these PI3Ks to adaptor proteins or Ras [35].

In vitro, class II PI3K enzymes have a lipid substrate specificity that is clearly distinct from that of class I and III enzymes. *In vitro,* class II PI3Ks can use PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 as substrates, with a strong preference for PtdIns > PtdIns(4)P >>> PtdIns(4,5)P_2 [34, 36]. It is not clear which lipids are made by class II PI3Ks inside cells: under experimental conditions whereby overexpression of p110α increases PtdIns(3,4)P_2/PIP3 levels, it has not been possible to demonstrate such an effect by overexpression of class II PI3Ks. A remarkable feature of some class II PI3Ks (PI3K-C2α and β) is that they can utilize Ca^{2+}/ATP for their *in vitro* lipid kinase activity [35, 37]. It is not clear, however, whether increases in cellular Ca^{2+} lead to the generation of 3′-PIs in cells.

Insulin, epidermal growth factor, platelet-derived growth factor, integrins, and a chemokine, MCP-1, can activate class II PI3K activity [37–40]. This has been demonstrated by measuring lipid kinase activity toward PtdIns in immunoprecipitates of class II PI3Ks. The mechanism by which these extracellular stimuli feed into these PI3Ks is unclear at the moment.

In contrast to class I PI3Ks which are mainly cytosolic, class II PI3Ks are predominantly associated with the membrane fraction of cells (low-density microsomal fraction, plasma membrane [35, 41]). Deletion of the C2 domain does not affect this subcellular localization [35].

Mammals have three class II isoforms (PI3K-C2α, β, and γ; encoded by three separate genes). PI3K-C2α and

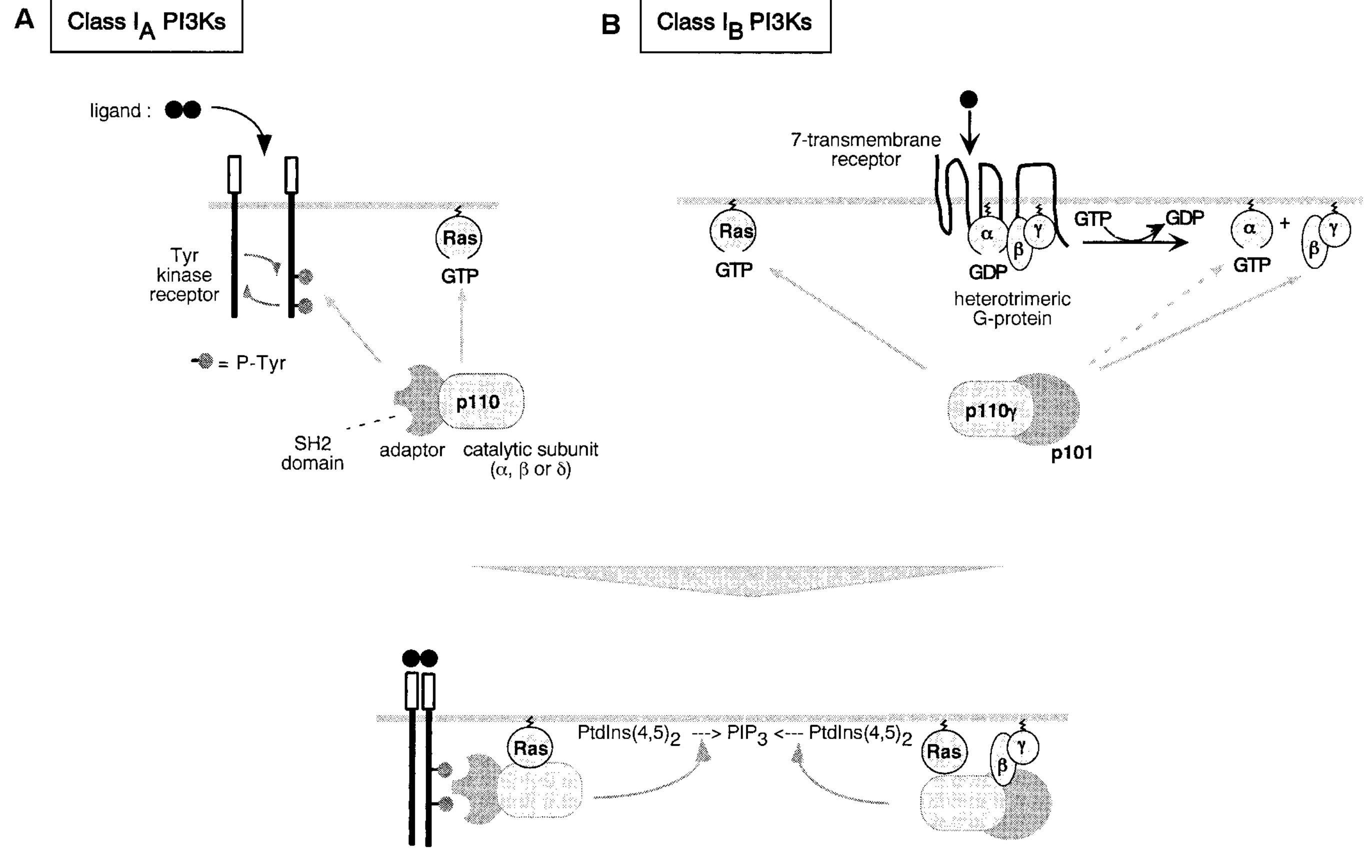


FIG. 3. Recruitment/activation of class I PI3Ks. (A) A receptor with intrinsic Tyr kinase activity is shown to dimerize upon binding of its cognate ligand and to transphosphorylate, creating recognition/docking sites for the SH2 domains of cytosolic class I_A PI3Ks. Apart from phosphorylating themselves, receptor Tyr kinases can also create phospho-Tyr docking sites in other molecules such as adaptor proteins (not shown). In some cases, the receptor or associated proteins are phosphorylated by cytosolic Tyr kinases (not shown). (B) Heterotrimeric G-proteins are made up of an α, β, and γ subunit, with the α subunit being the GDP/GTP binding module [174]. In the GDP-bound form, the α subunit interacts with the β and γ subunits to form an inactive heterotrimer that binds to serpentine receptors which span the membrane seven times. Ligand binding of these receptors induces a conformational change which results in an exchange of GDP for GTP on the α subunit, inducing its dissociation from both the receptor and $G\beta\gamma$ subunit (the β and γ subunits form an extremely tight dimer that can be considered as a functional monomer). The free $G\alpha$ and $G\beta\gamma$ subunits then interact with and modulate the activity of their target proteins such as p110γ. Note that class I PI3Ks do not necessarily interact simultaneously with Ras and phospho-Tyr or heterotrimeric G-protein subunits. It should be mentioned that the role, if any, of the Tyr phosphorylation of the class I_A adaptor subunit seen under some conditions is unknown at present.

β are fairly ubiquitous, in contrast to PI3K-C2γ which is mainly found in liver. A single type class II catalytic subunit is present in *Drosophila* (PI3K-68D) and *C. elegans* (F39B1.1). No class II PI3K family members are present in *S. cerevisiae* and no members have been found in *Dictyostelium, S. pombe,* or plants.

Class III PI3Ks

Class III PI3Ks are the homologues of the yeast vesicular protein-sorting protein Vps34p (reviewed in Ref. [42]). These PI3Ks can only use PtdIns as a substrate *in vitro,* and they are most likely to be responsible for the generation of a large fraction of the PtdIns(3)P in cells. The cellular levels of PtdIns(3)P remain fairly constant suggesting that the physiological processes in which class III PI3Ks are involved (see below) are not acutely triggered by cellular stimulation.

A single class III PI3K catalytic subunit has been identified in all eukaryotic species. Both in yeast and mammals, this catalytic subunit exists in complex with a Ser/Thr protein kinase (Vps15p in yeast, p150 in mammals). Vps15p and p150 are very homologous and N-terminally myristoylated. This lipid modification of Vps15p targets Vps34p to the membrane in yeast [43] and a similar role is anticipated for p150 in mammals.

Class IV PI3K-Related Kinases

Class IV PI3K-related kinases are a family of enzymes with a kinase domain that has significant ho-

mology to the kinase core domain of PI3Ks and PI4Ks. These proteins include among others TOR (target of rapamycin), DNA-PK (DNA-dependent protein kinase), and ATM (ataxia telangiectesia mutated). All available evidence indicates that the kinase domain of these molecules is involved in Ser/Thr protein kinase activity rather than in lipid phosphorylation: no lipid substrates for this class of kinases have been identified thus far.

PI3K INHIBITORS

Wortmannin and LY294002 are structurally unrelated, cell-permeable, low-molecular-weight compounds which are, at low doses, rather specific inhibitors of most PI3Ks. At higher concentrations, however, these compounds lose specificity (reviewed in Ref. [44]). In contrast to LY294002, wortmannin binds covalently to the PI3K catalytic subunits, a reaction that is competed by ATP and PtdIns(4,5)P_2 but not by PtdIns [45]. LY294002 is a competitive inhibitor of the ATP site [46].

All mammalian class I, II, and III PI3K members show a similar *in vitro* sensitivity to wortmannin and LY294002, apart from PI3K-C2α which is at least 10-fold less sensitive to these agents [36, 47].

The impact of wortmannin and LY294002 on class IV enzymes has been less well characterized. TOR and DNA-PK are inhibited by LY294002 to the same extent as PI3Ks, but are at least 10-fold less sensitive to wortmannin. The LY294002 sensitivity of ATM has not been reported, but as with TOR and DNA-PK it shows a reduced sensitivity to wortmannin compared to PI3K [48–51].

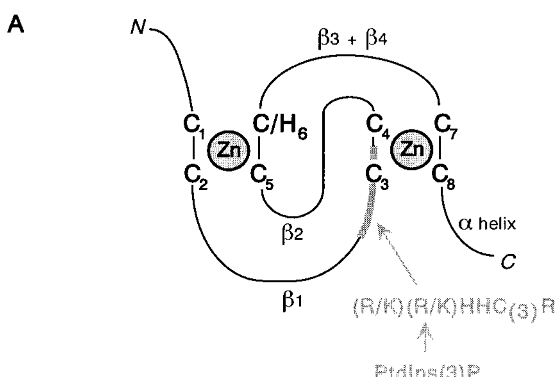
At present, evidence for an involvement of PI3Ks in a given biological system is obtained from treating cells with 5–20 μM LY294002 or 20–50 nM wortmannin. It should be mentioned that wortmannin, in contrast to LY294002, is rather unstable in aqueous solutions and also has a tendency to interact with serum proteins. This makes it difficult to assess the doses of active drug when applied to cells in tissue culture for periods longer than a couple of hours.

PI-BINDING DOMAINS

PIs in cells are specifically recognized by two structurally distinct lipid binding domains, the FYVE and PH domains. FYVE domains selectively bind PtdIns(3)P, whereas a subgroup of PH domains shows specificity for PtdIns(3,4)P_2 and/or PIP3. Specific binding of PIs to SH2 domains has been suggested [52] but recent structural and biochemical investigations argue against this notion [53].

A detailed characterization of these lipid binding domains has been made possible by the availability of synthetic lipids as well-defined tools [54, 55].

A



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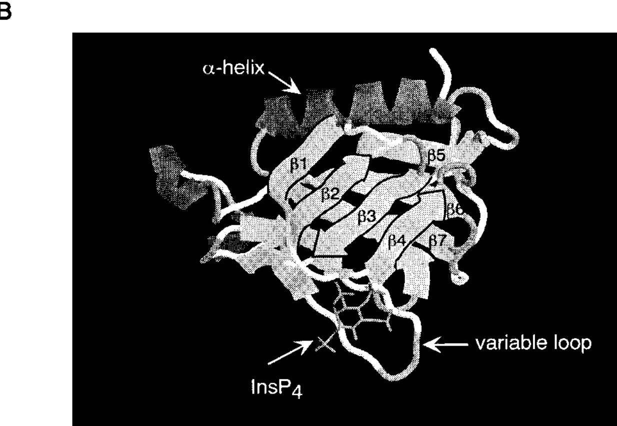

FIG. 4. (A) Schematic representation of the characteristic features of a FYVE domain. Indicated are the eight cysteine (C) residues that sequester Zn^{2+} (note that the sixth cysteine is replaced by a histidine in Vps27p). One Zn^{2+} ion is coordinated by the first and third pairs of cysteines, and a second Zn^{2+} is coordinated by the second and fourth pairs. The signature basic patch is shown as a bold line. Also shown is the location of the four β strands (1–4) and the C-terminal α-helix. (B) Ribbon diagram of the PH domain of Btk bound to Ins(1,3,4,5)P_4 (i.e., the head group of PIP3). The C-terminal α-helix, the two β-sheets (β1–4 and β5–7), and the three variable loops that interact with the inositol head group are shown.

FYVE Domain

The FYVE domain is a PtdIns(3)P-binding module, named after the first four proteins shown to contain it: Fab1p, YOTB, Vac1p, and *E*arly *E*ndosome *A*ntigen *1* (*EEA1*). It consists of ~60–80 amino acids with eight conserved cysteines that form two separate Zn^{2+} coordination centers. A characteristic basic amino acid motif [(R/K)(R/K)HH*C*R] surrounds the third cysteine and is involved in the binding of the inositol head group of PtdIns(3)P (Fig. 4A).

The structure of two FYVE domains has recently been determined [56, 57]. They are shown to consist of

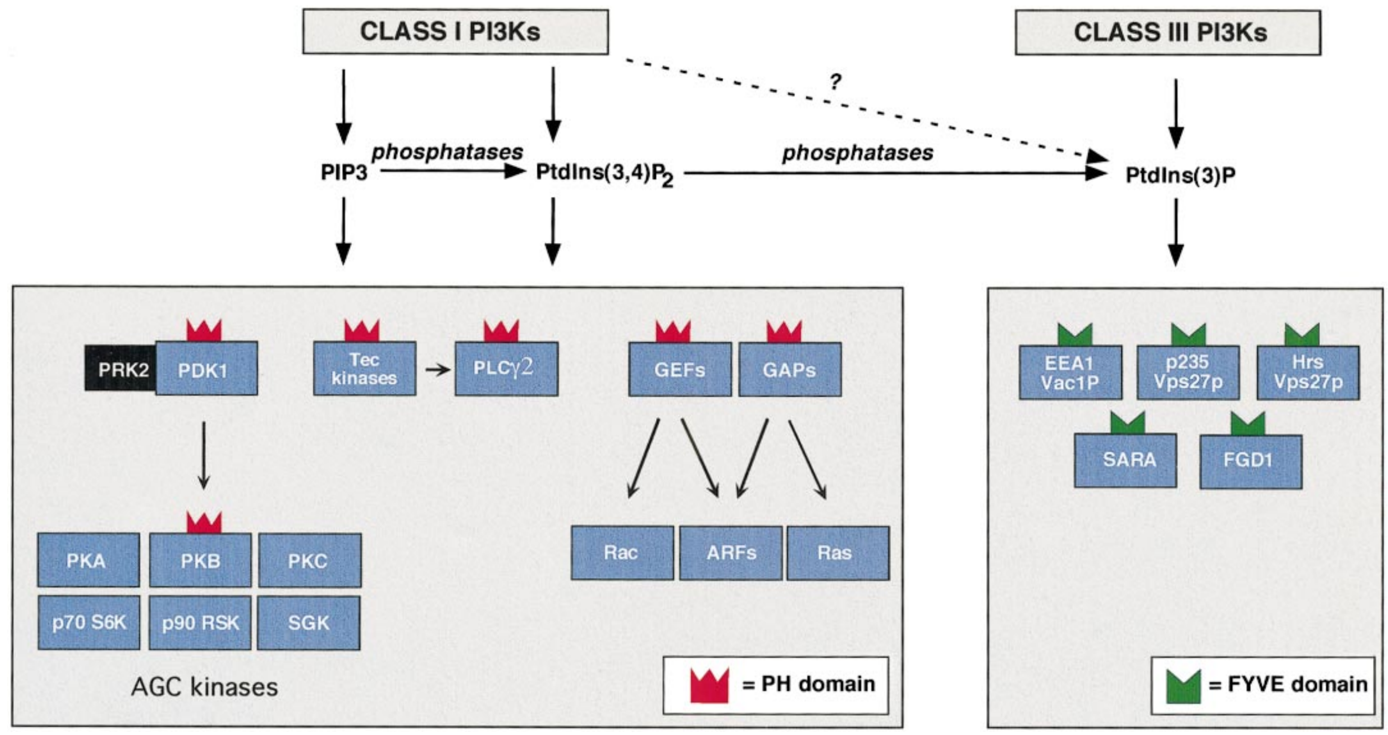

FIG. 5. Molecules with FYVE domains and 3′-PI-specific PH domains.

a long N-terminal loop, two small β sheets (referred to as "β hairpins" since each sheet is made up of two antiparallel strands), two Zn^{2+}-binding clusters, a small hydrophobic core, and C-terminal α-helix. The basic motif forms a charged patch on the surface of the FYVE domain. The structural data clearly reveal that only binding of PtdIns(3)P can be accommodated but not that of other lipids (such as PtdIns, PtdIns(4)P, or higher phosphorylated PIs; discussed in Refs. [58, 59]).

FYVE domains appear to be less widespread than PH domains. The yeast and *C. elegans* genomes reveal 5 and 11 different FYVE domain-containing proteins, respectively. To date, 22 different FYVE domain-containing proteins have been found in mammals (mouse, rat, human). Most FYVE domain-containing proteins have been implicated in membrane trafficking (Fig. 5; discussed below).

Pleckstrin Homology (PH) Domains

Pleckstrin homology (PH) domains are globular protein domains of about 100 amino acids that can bind phospholipids. A few PH domains also show a high-affinity interaction with the isolated, soluble inositol phosphate head groups of these lipids and certain PH domains may mediate protein–protein interactions [60–62].

PH domains have been identified in all eukaryotes in more than 100 different proteins including kinases, phospholipases, structural proteins, nucleotide-exchange factors, and adaptor proteins [60, 62, 63]. It is a common theme that lipid binding affects the localization, conformation, and/or activity of these proteins.

The core PH domain structure (Fig. 4B) is made up of two β-sheets (of four and three antiparallel β-strands), capped on one side by a C-terminal α-helix (that contains a highly conserved tryptophan residue). The two curved β-sheets are arranged at an angle of about 60° above each other and form a barrel-like structure. The loop regions that connect the β-strands are very variable in sequence and structure among PH domains. The inositol head group of the ligand is sandwiched between the loops at the end of the barrel distal from the C-terminal α-helix (the $\beta1$–$\beta2$ and $\beta3$–$\beta4$ loops). In some PH domains, the $\beta1$ and $\beta2$ strands possess two conserved basic residues that are essential for high-affinity binding of the phosphorylated inositol head group (see below).

Most PH domains bind PIs but only some do so with high affinity. Residues in PH domains that are most likely essential for high-affinity binding to PIs have recently been identified ([58, 64] and references therein). These residues lie at the N-terminus of the PH domain in a K-X_{8-13}-R/K-X-R-Hyd motif, where X is any amino acid and Hyd is a hydrophobic amino acid. The basic amino acids in this motif lie in the $\beta1$ and $\beta2$ strands and direct interactions with the inositol phos-

phate groups of PIs. PH domains that bind PIs with low affinity lack these residues [58]. *C. elegans, D. melanogaster,* and *Dictyostelium* (but not plants or yeast) possess this motif in some of their PH domains. It is therefore likely that plant and yeast PH domains will show low-affinity interaction with PIs and/or have other binding partners.

PH domains showing high affinity for PtdIns(3)P have not been reported, underscoring the importance of the FYVE domain as a crucial cellular sensor of PtdIns(3)P levels. A subset of PH domains preferentially binds PtdIns(3,4)P_2 and PIP3 over other PIs [58, 60, 62]. Most PH domains which interact with PIP3 also bind PtdIns(3,4)P_2 but frequently with much lower affinity. At present, there are no known examples of PH domains which interact only with PtdIns(3,4)P_2. However, the observation that some stimuli (such as ligation of integrins in platelets) induce large increases in PtdIns(3,4)P_2 levels without any increase in PIP3 [65] indicates that PtdIns(3,4)P_2 may induce signaling pathways distinct from those induced by PIP3, possibly by interaction with specific PH domains.

SIGNALING BY CLASS I PI3Ks

PtdIns(3,4)P_2/PIP3-binding PH domains are found in a diverse array of proteins (Fig. 5) including protein kinases (e.g., PKB, PDK1, Btk), nucleotide-exchange factors (e.g., Vav, GRP1, ARNO, cytohesin-1, Sos1, Tiam-1), GTPase-activating factors (e.g., GAP1^m, centaurins), phospholipases (e.g., PLCγ2), and adaptor proteins [60, 62]. In many cases, however, the exact lipid-binding specificity of these proteins has not yet been determined, but it is anticipated that many of them will indeed feature in PI3K signaling pathways.

Below, we summarize the signaling by proteins whose PH domains have been demonstrated to show a selectivity for binding 3′-PIs, namely the Ser/Thr kinases PKB and PDK1, the Tyr kinase Btk, and factors that control nucleotide turnover by ARF GTPases. Signaling that involves PI3K is also described elsewhere in this issue (contributions of Pessin, Thomas, and Hay).

PDK1, PKB, and AGC Kinases

PDK1 as an Activator of PKB

PKB derives its name from its homology to PKA and PKC. It is the cellular homologue of the viral oncoprotein v-*Akt,* hence its alternative name c-Akt or Akt. PKB consists of an N-terminal PH domain, a kinase domain, and a C-terminal regulatory region (Fig. 6). Two specific sites, Thr308 in the kinase domain and Ser473 in the C-terminal tail (numbering of human PKBα), need to be phosphorylated for full activation of this kinase (Fig. 6).

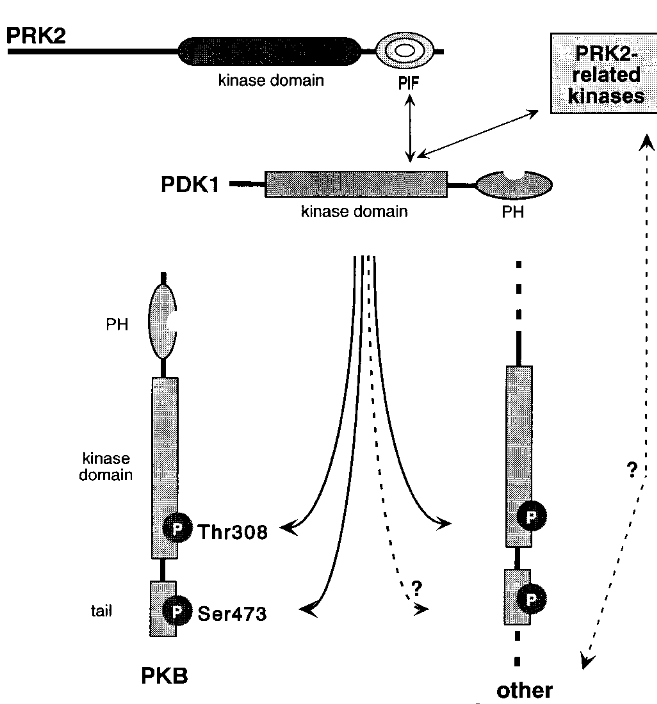

FIG. 6. Molecular architecture and functional interactions of PDK1, PKB, and AGC kinases. PIF, PDK1-interacting fragment, fragment of the C-terminus of PRK2 shown to interact with PDK1.

Since treatment of cells with PI3K inhibitors was known to abolish phosphorylation of Thr308 and Ser473, researchers set out to isolate the kinase(s) that could phosphorylate these sites in a 3′-PI-dependent manner. Together with genetic studies in *C. elegans* this work culminated in the identification of another PH domain-containing kinase, PDK1 (reviewed in Ref. [66]).

PDK1 was found to phosphorylate only Thr308, in a PtIns(3,4)P_2/PIP3-dependent manner (hence its name 3′-phosphoinositide-dependent kinase-1), and the tentative kinase responsible for Ser473 phosphorylation was named PDK2. Recent work indicates that PDK2 may in fact be a “modified” PDK1: when complexed with the PKC-related kinase PRK2 (or possibly an equivalent protein) PDK1 phosphorylates both Thr308 and Ser473, in a 3′-PI-dependent manner (Fig. 6). Further work is required to understand the physiological relevance of these findings and to find out how complex formation *in vivo* alters the biochemical characteristics of PDK1 (and possibly PKB).

PDK1 as an Activator of Other AGC Kinases

Amino acid sequences very similar to those surrounding Thr308 and Ser473 in PKB are conserved in all members of the AGC Ser/Thr protein kinases. This group includes PKA, PKG, PKC, and PKB isoforms as

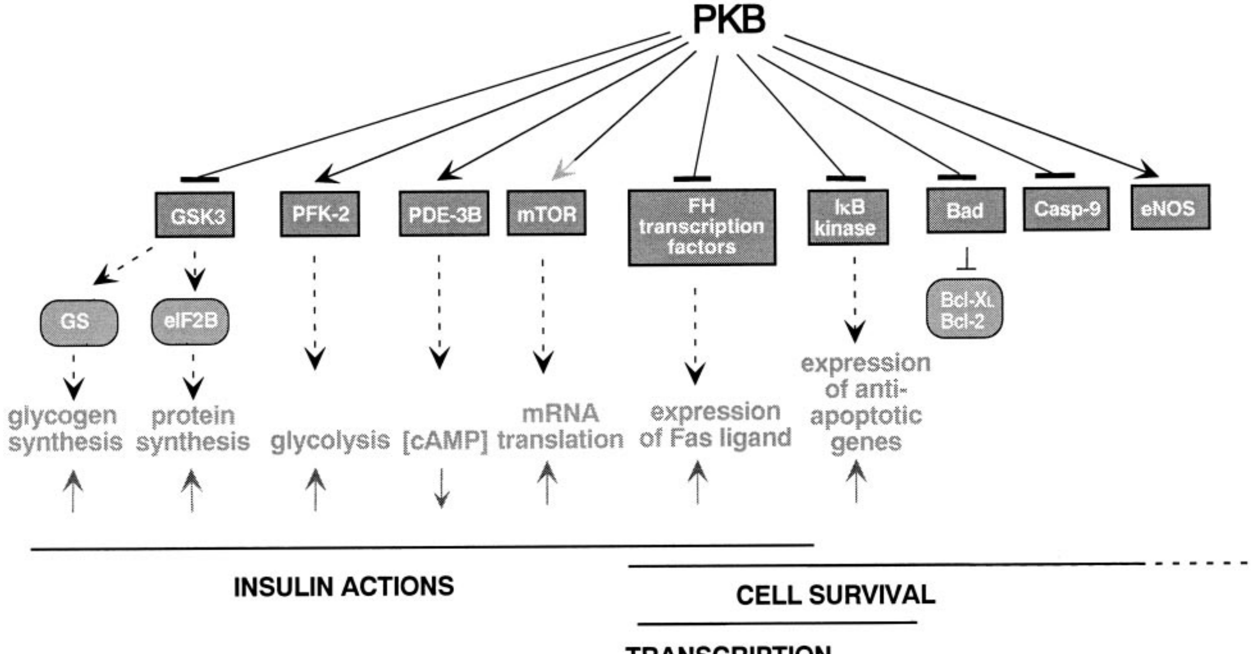

FIG. 7. Signaling downstream of PKB.

well as p70 S6-kinase, p90 ribosomal S6-kinases (p90RSKs), and serum- and glucocorticoid-induced protein kinase (SGK). Moreover, these homologous residues in AGC kinases become phosphorylated in cells upon activation under appropriate conditions. These findings suggested that the AGC kinases are activated by a mechanism analogous to that of PKB. In support of this hypothesis, PDK1 phosphorylates PKA [67], PKC isoforms [68, 69], SGK [70, 71], and p70 and p90 S6-kinase [72–75] at the residue equivalent to Thr308 of PKB (Fig. 6).

In vitro, this phosphorylation of AGC kinases by PDK1 is largely unaffected by PtdIns(3,4,)P_2/PIP3. This is somewhat remarkable given that *in vivo* the activation of p70 S6-kinase, SGK, and certain PKC isoforms is dependent upon PI3K activation (i.e., is blocked by PI3K inhibitors). It is possible that *in vivo* PDK1 is complexed with PRK2 or a related kinase which might render PDK1 sensitive to activation by PIP3/PtdIns(3,4)P_2 and possibly also allows it to phosphorylate the residues equivalent to Ser473 of the AGC kinases.

PKB Substrates

PKB actions have so far mainly been studied in the context of insulin signaling and the regulation of cell survival. Below we describe the seven direct targets for PKB phosphorylation that have been identified thus far (Fig. 7).

Targets of PKB in the regulation of cell survival. Overexpression of PKB delays cell death in many cellular model systems [76, 77]. This function of PKB might be important for cancer cells of pancreatic, ovarian, or breast origin in which PKB is frequently overexpressed [78, 79]. The observation of an anti-apoptotic role of PKB spurred a search for PKB substrates among the components of the cell death machinery. Two such targets, BAD (Bcl-2/Bcl-X_L antagonist, causing cell death) and caspase-9, have been reported.

BAD binds the antiapoptotic proteins Bcl-2 and Bcl-X_L and thereby prevents them from exerting their antiapoptotic function. When phosphorylated on Ser112 or Ser136, BAD no longer interacts with either Bcl-2 or Bcl-X_L, allowing them to inhibit apoptosis. PKB can phosphorylate BAD on Ser136, and this could be one way by which PKB contributes to cell survival ([80] and references therein]. It is important to mention, however, that not all cell types express BAD and that cell survival can be regulated independently of both PKB activation and BAD phosphorylation [81, 82]. Similarly, specific inhibition of class I_A PI3Ks (and thus PKB) by inducible expression of a dominant-negative PI3K does not necessarily lead to apoptosis [83]. Other kinases with possibly equal importance as PKB in BAD phosphorylation are PKA (which can phosphorylate Ser112 [84]) and MAP kinases [81].

Caspase-9 is a protease crucial in the initiation and possibly later stages of apoptosis [85, 86] which can be phosphorylated and inhibited by PKB [87]. It is not yet

clear how important and/or general this is for PKB-mediated regulation of apoptosis.

Recently, two transcription-based mechanisms by which PKB can interfere with cell death have been reported. The first is via members of the forkhead (FH) family of transcription factors (FKHR, FKHRL1, and AFX) which have been shown to be direct targets for phosphorylation by PKB [88–92]. In serum-starved cells, these FH transcription factors predominantly reside in the nucleus, whereas upon cellular stimulation they are mainly found in the cytosol. There is good evidence to suggest that PKB is a crucial determinant of this differential subcellular localization. The currently held view is that phosphorylation of FH transcription factors by PKB promotes their export from the nucleus to the cytosol where they may interact with the cytosolic 14-3-3 proteins, effectively holding them in the cytoplasm, away from their target genes in the nucleus [88, 89, 91]. Phosphorylation of nuclear targets by PKB is consistent with its documented translocation from the cytosol upon activation [93, 94]. FH transcription factors have been implicated in expression of the Fas ligand, which can induce cell death upon autocrine or paracrine production: upon phosphorylation by PKB, FH transcription factors are retained in the cytosol and therefore the Fas ligand is not expressed, allowing the cells to survive [88].

PKB can also associate with and activate I-κB kinases (IKKs) that regulate the activity of the NF-κB (nuclear factor κB) transcription factor [95–98]. When bound to its cytosolic inhibitor I-κB, NF-κB is inactive as a transcription factor. Upon phosphorylation of I-κB by IKKs, the inhibitor is degraded, allowing NF-κB to move to the nucleus and activate the transcription of antiapoptotic proteins [99]. The mechanism by which PKB activates IKKs is not entirely clear, but one study [97] has implicated a direct phosphorylation by PKB of the α form of the IKKs.

More PKB targets with an antiapoptotic role will undoubtedly be uncovered. It is important to mention that the regulation of metabolic pathways by PKB (see below) is likely to be equally important in the control of cell survival as the mechanisms mentioned above.

Targets of PKB in insulin signal transduction. Many of the responses of a mammalian cell to insulin are conveyed via PKB [100–107]. Upon stimulation of cells by insulin, PKB has been shown to phosphorylate five important components of the insulin signal transduction cascade. PKB phosphorylates and inhibits glycogen synthase kinase 3 (GSK3), a kinase that is constitutively active in resting cells [108]. Its substrates include metabolic enzymes (e.g., glycogen synthase) and translation factors (e.g., eIF2B, an exchange factor for the translation initiation factor eIF2) which are inhibited upon phosphorylation by GSK3. The glycogen synthase and eIF2 pathways become activated upon ligand-stimulated phosphorylation of GSK3 [108, 109]. PKB also phosphorylates and activates the cardiac isoform of 6-phosphofructo-2-kinase (PFK-2) [110]. PFK-2 catalyzes the synthesis of fructose 2,6-bisphosphate, which is a potent stimulator of 6-phosphofructo-1-kinase, a controlling enzyme of glycolysis. The activation of PFK-2 by insulin in heart is thus partly responsible for the stimulation of glycolysis in this tissue. Another target that is activated by PKB is phosphodiesterase-3B (PDE-3B), an enzyme important in the regulation of the cellular concentrations of the second messenger cAMP [111]. The most recent addition to the list of PKB targets is the mammalian target of rapamycin (mTOR), which is important in the regulation of protein translation [112]. The impact of this phosphorylation on the activity of mTOR has not been firmly established. The PKB-mediated phosphorylation of FH transcription factors (see above) is most likely also involved in the regulation of gene expression by insulin [90, 92, 113].

Endothelial nitric oxide synthase. Endothelial nitric oxide synthase (eNOS) phosphorylation by PKB results in an activation of this enzyme [114, 115]. Sustained production of nitric oxide by endothelial cells has been implicated in many biological effects, such as gene regulation and angiogenesis.

Tec Family Tyr Kinases and PLCγ2

Tec kinases are nonreceptor Tyr kinases with a PH domain N-terminal to their SH3/SH2/kinase core structure which they have in common with the Src family kinases. This PH domain seems to substitute for the N-terminal lipid modification motif necessary for membrane targeting of Src. Tec family members include among others Btk (Bruton's tyrosine kinase) and Itk (inducible T-cell kinase). The Btk PH domain has been shown to bind PtdIns(3,4)P_2/PIP3 with high affinity and selectivity [60, 61], and there are indications that the Itk PH domain may also bind 3′-PIs but its detailed lipid binding features await characterization [116].

Btk is critical for B cell development and function, and germline mutations in the Btk gene have been shown to cause immunodeficiency. Several of these mutations have been found to map to the PH domain of Btk [117].

There is some evidence to suggest that Tec kinases might be activated in a similar way to PKB. The PH domain is believed to mediate the translocation of Tec kinases to the plasma membrane where further activating phosphorylations occur. These phosphorylations on Tyr and Ser might be mediated by Src family kinases or PDK1-related enzymes.

One of the proposed Btk targets is PLCγ2 [118]

which also has a PIP3-specific PH domain [119]. Both cytosolic enzymes might be brought into contact which each other by a PI3K-induced cotranslocation to the plasma membrane (discussed in Refs. [118, 120]).

GTP/GDP-Exchange Factors and GTPase-Activating Proteins

Small GTPases cycle between an inactive GDP-bound and active GTP-bound form. This cycle is regulated by GEFs (GTP/GDP-exchange factors) and GAPs (GTPase-activating proteins). GEFs catalyze the release of GDP and subsequent binding of GTP while GAPs stimulate the slow intrinsic GTPase action of these proteins, converting them from active to inactive status. Different families of GTPases are affected by distinct families of GEFs and GAPs.

GEFs

PH domains are found in all GEFs specific for the Rho family of GTPases (that includes Rho, Rac, and cdc42) and in a subclass (namely those that are insensitive to the drug brefeldin A) of GEFs specific for ARF-GTPases. These ARF-GEFs show an exquisite specificity for binding PIP3. The lipid binding specificity of most Rho-GEFs has not yet been investigated in detail.

GEFs for Rac

Inhibition of PI3K blocks the actin reorganization and chemotaxis stimulated by Tyr kinases. This inhibition can be overcome by introduction of constitutively active Rac, suggesting that Rac acts downstream of PI3K in signaling pathways leading to actin reorganization. This conclusion is corroborated by the observation that PI3K activation increases cellular Rac-GTP levels. The precise mechanism through which PI3Ks activate Rac has not been elucidated, but some data suggest that Vav, a GEF for Rac, can be directly stimulated by 3′-PIs [121]. Tiam1, another Rac-GEF, has also been shown to bind PIP3 but the functional impact of this binding is not clear at the moment [62].

GEFs for ARF GTPases

ARF proteins play a role in vesicular membrane trafficking in several intracellular compartments and, upon exchange of GDP for GTP, translocate from the cytosol to intracellular membranes where they induce the docking of vesicle coat proteins. Three ARF-GEFs have been shown to be regulated by PI3K, namely GRP1 (general receptor for phosphoinositides), ARNO (ARF nucleotide binding site opener), and cytohesin-1 [122, 123]. They all contain PH domains that preferentially bind PIP3 over PtdIns(3,4)P_2 and other PIs. These cytosolic proteins move to the plasma membrane in response to PIP3-generating signals and are redistributed to the cytosol if PIP3 production is inhibited [124–128].

Cytohesin-1 was isolated as a protein that binds to the intracellular portion of β2-integrin and regulates integrin-mediated adhesion of leukocytes. It is unresolved how the ARF-GEF activity of cytohesin-1 is linked with its function in cell adhesion.

GAPs

The centaurins [129] and the GAP1^{m} and GAP1^{IP4BP} proteins [130] specifically bind PIP3 or its soluble Ins(1,3,4,5)P_4 head group *in vitro.* Centaurin-α and GAP1^{m} have been shown to be PIP3-binding proteins inside cells [125, 131].

The centaurins have homology to yeast ARF-GAPs and consistent with this, centaurin-α1 can complement a yeast strain deficient in the ARF-GAP Gcs1 [125].

GAP1^{m} and GAP1^{IP4BP} are Ras-GAPs. The roles of 3′-PIs in the function of these proteins is unclear at present: PIP3-induced recruitment of GAP1^{m} from the cytosol to the plasma membrane did not appear to result in enhancement of its basal Ras-GAP activity [131].

SIGNALING BY CLASS II PI3Ks

As mentioned above, it is not clear whether *in vivo* these kinases produce the lipids that induce signaling via PH or FYVE domains. The association of class II PI3Ks with cellular membrane compartments might suggest participation of these enzymes in sorting events or vesicle formation, and it is likely that production of 3′-PIs at these specific locations will be crucial to the function of class II enzymes.

Functional studies in mammals are complicated by the presence of multiple PI3K isoforms. In flies there is only one isoform of each PI3K class [34]. Recent work in this organism has provided strong evidence that class I and II PI3Ks affect distinct biological processes: when overexpressed in the precursor to the wings and thorax, the class II PI3K affects pattern formation but not growth in these adult organs, whereas the class I enzyme only affects growth and organ size [132, 133].

SIGNALING BY CLASS III PI3Ks

PIs in general play a critical role in the regulation of eukaryotic membrane traffic [134] and this is most likely also the case for all 3′-PIs. Thus far, however, this field has primarily focused on PtdIns(3)P. This is related to the fact that yeast which lacks class I and II PI3Ks has been the main model system for investigations into the role of PI3K in membrane traffic.

Non-class III PI3Ks are likely to impinge on stimulus-induced membrane traffic, a phenomenon that

might be intimately linked with and essential for the proper signaling via cell surface receptors [135, 136]. This notion is strengthened by the recent observation that PIP3 modulates the function of the ARF proteins that are key players in vesicle traffic (see above). Obviously, PtdIns(3)P can be generated from PIP3 by the action of lipid phosphatases, and class I PI3Ks can thus contribute to signaling via FYVE domains (Fig. 5). Using a microinjection approach, the class I PI3K p110α has been implicated in transferrin recycling [137]. p110β/p85α can associate with Rab5, a small GTPase which regulates the fusion of early endosomes [138]. Class I_A PI3Ks appear to be dispensable for receptor-mediated endocytosis but play a role in postendocytic steps of receptor trafficking, namely in the targeting of receptors to the lysosomes for degradation. This conclusion is based on the finding that PDGF receptor mutants that do not bind class I_A PI3Ks still internalize after ligand binding, but are no longer degraded [139].

The fact that class III-mediated membrane traffic events are equally important in the regulation of extracellular signaling is underscored by the observation that microinjection of antisera to mammalian Vps34p blocks insulin-stimulated proliferation [137]. Likewise, PtdIns(3)P-mediated recruitment of the SARA (Smad anchor for receptor activation) protein to the TGF-β receptor is essential for signaling via this receptor (see below and Refs. [140, 141]).

The biology of class III PI3Ks has been extensively reviewed recently [142] and will only be summarized here in the context of the proteins that contain a FYVE domain.

In yeast, Vps34p is essential for the traffic of proteins from the Golgi complex to the vacuole, the equivalent of the mammalian lysosome. A similar role for the mammalian Vps34p has been invoked based on studies using PI3K inhibitors. The FYVE domains that bind the Vps34p lipid product are mainly found in proteins that play a role in membrane traffic events along the secretory and endocytic pathways (Fig. 5) and include the mammalian proteins EE1A, p235, and Hrs and their putative respective yeast orthologs Vac1p, Fab1p, and Vps27p. A role in membrane traffic has not been established for SARA and FGD1, two other mammalian FYVE domain-containing proteins.

EE1A is an effector of Rab5 which itself also binds human Vps34p and p85α/p110β [138, 143]. Vac1p, the yeast EE1A ortholog, equally binds to Rab5-GTP and interacts with several other proteins involved in the regulation of vesicle docking and fusion [144]. Fab1/p235 are PtdIns(3)P-5′-kinases whose activity might also be triggered by extracellular stimulation [7, 143, 145, 146]. Fab1p in yeast has been implicated in the formation of multivesicular endosomes which are structures required for sorting of cell surface receptors for downregulation and degradation within lysosomes. The functional inactivation of Fab1p in yeast is also associated with an expanded vacuole [145]. Vps27p plays a role in endosome maturation processes such as receptor recycling and multivesicular body formation [142, 147].

SARA (Smad anchor for receptor activation) is a protein that recruits isoforms of the smad transcription factors to the TGFβ receptor [140, 141]. FGD1 is a Rho-GEF which is defective in the human disease faciogenital displasia. It is not clear at the moment how SARA and FGD1 link to the other components of the PtdIns(3)P pathway.

PI3K PROTEIN KINASE ACTIVITY

In addition to their lipid kinase activity, class I and III (but not class II) PI3Ks possess an *in vitro* auto- or inter-subunit protein phosphorylation capacity. There is some evidence that exogenous substrates, such as IRS, could equally function as substrates for PI3K phosphorylation [148]. Two recent reports indicate that PI3K protein kinase activity can also operate in a cellular context. Using phospho-specific antisera against the p110δ autophosphorylation site, we found that this residue becomes phosphorylated in endogenous p110δ in cells, in a stimulus- and wortmannin/LY294002-dependent manner [148]. Class I_B PI3K protein kinase activity has been shown to activate MAPK in a transient transfection system [149]. In this study, the authors made use of p110γ mutants that could no longer phosphorylate lipids but had retained their protein kinase activity. The direct target for protein phosphorylation in this system has not been firmly established, and further work is required to assess whether this activity is also operative under more physiological conditions. *In vitro* protein kinase activity is a distinguishing feature among the PI3K isoforms and may contribute to isoform-specific functions in cells (see below). It is therefore important to identify physiological protein kinase targets of PI3Ks.

CLASS I_A PI3K ISOFORM-SELECTIVE ACTIVITIES

Mammals have multiple class I_A adaptors and catalytic subunits. This renders this signaling system not amenable to (stable) overexpression of single adaptor or p110 genes: overexpression of adaptor proteins has a dominant-negative effect on the recruitment of endogenous class I_A PI3K to receptors and appears to be rather toxic for cell lines. Stable overexpression of wild-type p110 subunits without adaptors has not been reported thus far. This could be due to toxicity but might also be related to the fact that p110 proteins appear to be unstable in the absence of adaptor proteins [150]. Thus, rather than overexpression approaches, PI3K

gene-targeting studies in mice and microinjection of PI3K antibodies into cells have begun to uncover the roles played by individual class I adaptors and catalytic subunits.

Class I_A Gene Knockouts

Mammals have three class I_A adaptor genes: p85α, p85β, and p55γ, and alternative splicing of the p85α gene gives rise to at least four isoforms (p50α, p55α, p55α_i, and p85α_i [11]). Gene targeting of the first exon of p85α still allows synthesis of its p50α and p55α isoforms. These mice are viable but develop a B cell immunodeficiency [151]. No impact on T cell function was notable, possibly due to a compensatory overexpression of the p50α isoform, which was not seen in B cells [151]. These mice also have an increased responsiveness to insulin, providing evidence for a role of PI3K in glucose homeostasis *in vivo* [152].

Knockout of p85α and all its splice variants results in death of the mice within days after birth, which is in contrast to p85β knockout mice that are viable and for which no phenotype has been reported thus far [153, 153a]. The contrasting findings for these two adaptors possibly relate to the fact that p85α is generally the most abundant class I_A adaptor in cells. The underlying cause of death of p85α knockout mice is not clear, but might be related to the reduced expression of all p110 isoforms. Using a method that allows assessment of the impact of gene targeting specifically in lymphocytes in chimeric mice, it was found that B cell development and function were impaired in the absence of p85α gene products. Remarkably, T cells appeared normal in the absence of p85α [153]. p85β levels in the latter cells remained unchanged but the impact on p55γ expression has not been reported.

Class I_A adaptors show no selectivity for binding specific p110 isoforms. Gene targeting of these adaptors is therefore expected to have an impact on the function of all p110s; hence, p110 gene knockout studies are still essential to delineate the function of individual catalytic subunits. Disruption of the p110α gene in mice has been found to result in embryonic lethality approximately halfway through term [154]. Remarkably, p85α was found to be overexpressed in these embryos. This is expected to have a dominant-negative effect on all p110s and may contribute to the lethal phenotype. This notion is underscored by the finding that cells from embryonic tissue explants of these mice failed to grow in culture.

From the data mentioned above, it is clear that knockout of class I_A adaptor subunits affects the expression of the p110 subunits and vice versa. Gene-targeting approaches that avoid disturbances of the adaptor/catalytic subunit ratios in cells will be essential in order to be able to unequivocally interpret the results of such studies.

Microinjection of p110 Antibodies

The results of PI3K antibody microinjection experiments suggest that p110 isoforms have distinct functions. Roche *et al.* showed that p110α is involved in cell proliferation induced by PDGF, insulin, and EGF but not in bombesin- or LPA-mediated mitogenic responses [33, 155]. p110β appears to be necessary for LPA- and insulin-mediated but not PDGF-mediated mitogenic responses [33]. We found that CSF1-induced DNA synthesis in macrophages is dependent on p110α but not on p110β or p110δ. Conversely, p110β and p110δ, but not p110α regulate the actin cytoskeleton and cell migration in these cells [156]. Recent studies in endothelial cells revealed that actin reorganization by PDGF is controlled by p110α but not p110β, whereas the converse is true for insulin where p110β but not p110α plays the main role [157]. In insulin receptor-overexpressing CHO cells, however, a role for p110α in insulin-stimulated ruffling has been demonstrated [137]. Cell type-dependent effects should be taken into account when interpreting these studies especially when for example polarized epithelial cells (with asymmetrically distributed signaling molecules) are compared with cell types such as leukocytes. It is probably equally important in this type of study to use endogenous and not overexpressed receptors, as overexpression can saturate normal compartments and put signaling components in abnormal places.

These observations suggest (at least) two layers of control of PI3K function in cells: differences intrinsic to the PI3K isoforms are combined with a receptor-dependent control and direction of PI3K action. This would mean that for example PDGF integrates p110α in signaling cascades other than insulin. Different receptors have distinct routing and recycling mechanisms which will target PI3Ks and their activities to specific subcellular localizations and compartments [135, 158].

An example of an intrinsic difference among PI3Ks is their isoform-specific protein kinase activity [148], but its relevance for isoform-specific functions is unclear as yet. There is increasing evidence for a p110-specific association with cellular proteins: p110β but not p110α binds to Rab5 [138], and p110β and p110δ (but not p110α) interact with Ras under specific conditions of cellular stimulation [159]. p110β but not p110α becomes recruited to glucose transport vesicles upon insulin stimulation [160], which is possibly related to the specific association of p110β with Rab5 (see above). The class I_A adaptors are very diverse in their N-terminal regions which may contribute to differences in their protein binding partners, subcellular distribution, and level of activation of the catalytic subunits in

response to stimulation [161–165]. Some receptors or their adaptors (such as insulin receptor substrates) appear also to have a binding preference for specific PI3K adaptors [165, 166].

CONCLUSIONS AND FUTURE PROSPECTS

Significant advances have recently been made in our understanding of the class I and III PI3Ks and the mechanism by which membrane-bound lipids convey signals to the cytosol. 3′-PI-binding domains have been uncovered in a wide variety of proteins, and more of such proteins will undoubtedly follow. It will be a challenge to understand how signaling by such a multitude of proteins is integrated inside cells. Fundamental to such understanding will be a robust comparison of the lipid binding characteristics of PH domains using more standardized approaches of lipid presentation.

An important outstanding question is the function of the class II PI3Ks and the signals that regulate their activity. Likewise, the roles played by each of the mammalian PI3K isoforms in the organism need to be uncovered, and physiological targets of the protein kinase activity of PI3K isoforms remain to be identified.

The importance of deregulated PI3K signaling in disease is becoming increasingly apparent. This is underscored by the finding that PTEN, an important tumor suppressor, acts directly on the PI3K lipid products ([9] and Hay, this issue) and that components of the PI3K signal transduction pathways are mutated or overexpressed in certain cancers [78, 79, 167–172]. There is increasing evidence for a deregulation of PI3K action in diabetes, and it is anticipated that more implications of PI3Ks in disease will be uncovered by techniques such as array-based PI3K gene profiling. It is hoped that ongoing efforts to develop isoform-specific PI3K inhibitors will allow pharmaceutical intervention with clinical benefit to humans.

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