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Phosphoinositides and signal transduction

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Abstract. Phosphoinositides comprise a family of eight minor membrane lipids which play important roles in many signal transducing pathways in the cell. Signaling through various phosphoinositides has been shown to mediate cell growth and proliferation, apoptosis, cytoskeletal changes, insulin action and vesicle trafficking. A number of advances in signal transduction in the last decade has resulted in the discovery of a growing list of proteins which directly interact with high affinity and specificity with distinct phosphoinositides. Equally important, a number of phosphoinositide binding domains such as the pleckstrin homology domain have emerged as critical mediators of phosphoinositide signaling. Here, recent advances in phosphoinositide signaling are discussed. The aim of this review is to highlight particularly exciting advances made in the field over the last few years. The regulation of phosphoinositide metabolism by lipid kinases, phosphatases and phospholipases is reviewed, and considerable emphasis is placed on phosphoinositide-binding proteins. Finally, the role of these lipids in regulating signaling pathways and cell function is described.

Key words. Phosphoinositides; phosphoinositide kinases; phosphoinositide phosphatases; signal transduction; PH domains; protein kinases.

Introduction

The pioneering work of Hokin and Hokin in the mid 1950s was the first foray into phosphoinositide signaling for biologists and biochemists [1]. Their seminal discoveries indicated that phosphoinositides, particularly PtdIns-4-P and PtdIns-4,5-P_2 as well as the enzymes which are responsible for their synthesis, directly participate in agonist-dependent signal transduction in cells. In the last 20 years there have been many fundamental discoveries which have brought phosphoinositide signaling to the very forefront of current biological and biomedical research. Consequently, in the present day it is difficult to think of a physiological process which does not utilize some form of phosphoinositide-dependent signal transduction. Initial work was focused in the discovery and characterization of the enzymes responsible for phosphoinositide metabolism, including phosphoinositide kinases, phosphatases as well as phospholipases. Although significant discoveries are still being made with respect to these enzymes, part of the focus has recently switched to the downstream targets of the phosphoinositides themselves, and how they mediate signaling cascades. Success in this area has been due to the availability of the clones of many of the phosphoinositide kinases for in vivo studies as well as the availability of potent and specific inhibitors of these enzymes [e.g. wortmannin and LY294002, phosphoinositide 3-kinase (PI 3-K) antagonists] and in particular, the synthesis of phosphoinositide analogues for in vitro studies. The most recent advances in the field are coming from various genetic knockouts of the phosphoinositide kinases in various organisms.

The fact that phosphoinositides are minor constituents of cell membranes (PtdIns typically comprises approximately 10% of total membrane lipid) immediately indicated that these lipids are not likely to provide a structural role, but rather exert specific regulatory functions such as agonist-stimulated signal transduction. It is now clear that phosphoinositides initiate signaling by specifically interacting with a large number of proteins. These specific and high-affinity interactions can have several con-

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sequences depending on the nature of the protein. They can relocalize the protein from one area of the cell to another, typically from the cytosol to the inner leaflet of the plasma membrane. Also, the lipid-protein interaction can induce a conformational change in the protein, which can also result in modulation of its activity. Alternatively, phosphoinositide-protein interactions can have a profound effect on the lipid itself, such as phosphorylation or dephosphorylation or protection from phospholipase-catalyzed hydrolysis. The net result from various combinations of these effects is that the phosphoinositide-protein interaction can initiate downstream signaling cascades leading to cell growth and proliferation, death, differentiation, secretion and vesicle trafficking, motility and intracellular metabolism.

Thus, one aim of this review is to highlight some of the major discoveries made in the last 10 years on phosphoinositide signaling. Recently there have been many excellent reviews which have dealt with specific areas of research in the field, such as the role played by PI 3-K lipids [2, 3], the structure and function of the phosphoinositide phosphate-kinase (PIP-kinase) [4, 5] and PI 3-K families [6]. Here, we will summarize major discoveries in the field and pay particular attention to progress made in the last few years. Specifically, regulation of phosphoinositides will be discussed, with emphasis placed on the role played by phosphoinositide kinases. The lipid protein interaction will be discussed by highlighting several proteins and protein domains which interact with phosphoinositides, particularly PtdIns-4,5-P_2 and PtdIns-3,4,5-P_3. We will pay considerable attention to the pleckstrin homology (PH) domain, which has emerged as a major phosphoinositide binding domain. Finally, there will a be a discussion on the major signaling pathways utilized by phosphoinositides and their impact on biology and disease.

Synthesis and degradation of phosphoinositides

Phosphoinositide Kinases

The immediate precursor of all phosphoinositides is phosphatidylinositol (PtdIns, fig. 1A). The concerted action of phosphoinositide kinases acting at either the 3, 4 or 5 position of PtdIns results in the generation of seven additional lipids, as depicted in figure 1. The canonical pathway utilizes PtdIns as a substrate for PI 4-kinases, thus generating PtdIns-4-P which in turn serves as a substrate for PtdIns-4-P 5-kinases to yield PtdIns-4,5-P_2. This then in turn is hydrolyzed by phospholipases to generate the second messengers Ins-1,4,5-P_3 (IP_3) and diacylglycerol (DAG). Two families of PtdIns 4-kinases exist, types II and III. α and β subtypes of the type III PtdIns 4-kinase have been purified and cloned, and type II enzymes have been purified to homogeneity. Although PtdIns 4-kinases have a ubiquitous distribution in mammals, there is evidence of distinct subcellular distribution, indicating that localized pools of PtdIns-4-P are present in the cell membranes. For a comprehensive review on PtdIns 4-kinases see Balla [7]. Although the major route for PtdIns-4,5-P_2 synthesis is phosphorylation of PtdIns-4-P at the 5 position by PIP kinases, the recent discovery of a previously unknown lipid, PtdIns-5-P, indicated that additional routes of synthesis exist [8]. As shown in figure 1B, PtdIns-5-P can either be generated by the action of a PtdIns 5-kinase, or by dephosphorylation of PtdIns-4,5-P_2 by a 5OH phosphatase such as SHIP (SH2-containing inositol 5′ phosphatase). Although the latter reaction occurs in vitro [8], it is not known whether this represents a physiologically relevant mechanism for the generation of PtdIns-5-P. Similarly, a PtdIns 5-kinase could exist which would utilize PtdIns as substrate to generate PtdIns-5-P; a type I PtdInsP kinase has been shown to carry out this reaction in vitro [8], but again it is not known whether this is physiologically relevant.

In summary, the action of PtdInsP kinases as well as 5OH phosphatase(s) generates the important lipid second messenger, PtdIns-4,5-P_2. The enzymes which are responsible for the production of PtdIns-4,5-P_2 in cells have recently been revisited with respect to their precise substrate specificities. The major pathway for the generation of PtdIns-4,5-P_2 in cells is from the action of PtdIns-4-P 5-kinases on PtdIns-4-P, although it is conceivable that discrete pools of this lipid are made at different cell locations by the action of PtdIns-5-P 4-kinases on PtdIns-5-P. Thus, PIP kinases as they are now known fall into two distinct classes, types I and II, each with three distinct subfamilies (named α, β and γ), and curiously they do not share any obvious sequence homologies with other phosphoinositide kinases [5]. Originally, the only known homologues of PIP kinases were the yeast proteins Fab1p and Mss4p [9, 10], although now there are over 20 proteins in sequence databases which have similarities with PIP kinases. Type I PIP kinases preferentially use PtdIns-4-P to generate PtdIns-4,5-P_2 by phosphorylation at the 5OH position (fig. 1). In addition to this activity, type I enzymes can also phosphorylate the PI 3-K lipid PtdIns-3-P to generate both PtdIns-3,4-P_2 and PtdIns-3,5-P_2 in vitro [8, 11]. Thus, these are dual-specificity enzymes capable of phosphorylating both the 4OH and 5OH positions. Recent work from a number of laboratories has revealed that type II PIP kinases, rather than phosphorylating PtdIns-4-P, actually prefer PtdIns-5-P as a substrate and phosphorylate the 4OH position to generate PtdIns-4,5-P_2 [8]. As with the type I enzymes, type II PIP kinases can also use PtdIns-3-P to generate PtdIns-3,4-P_2. Thus, type II enzymes appear to be true 4OH kinases unlike type I enzymes which seem more promiscuous. Again, whether this specificity is retained in cells remains to be established.

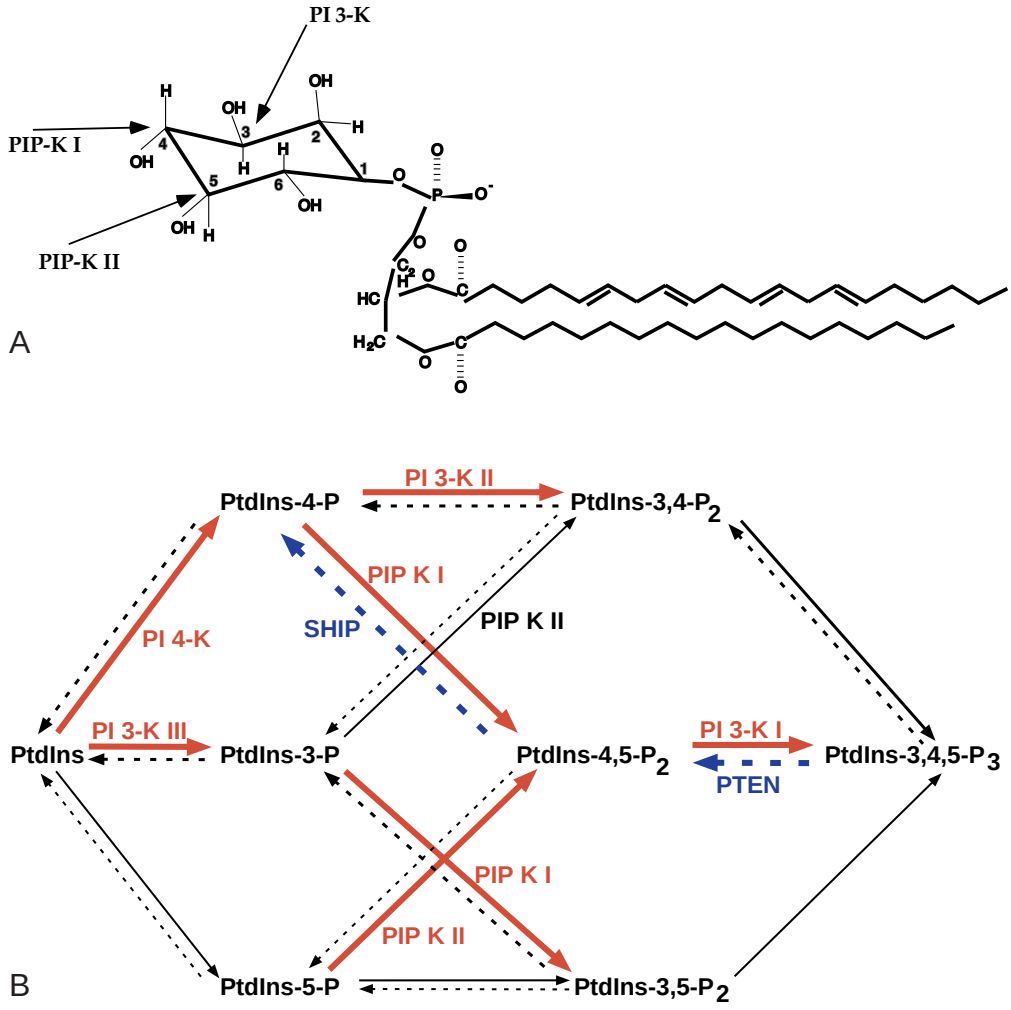

Figure 1. (*A*) Phosphatidylinositol. The structure shows PtdIns, which contains a *myo*-inositol headgroup connected to a diacylglycerol via a phosphodiester linkage. The fatty acid moiety is typically stearoyl-arachidonyl. The inositol head group can be phosphorylated at one of three currently identified positions, 3OH, 4OH and 5OH, by PI 3-Ks and PIP kinases (types I and II), respectively. (*B*) Phosphoinositide metabolism. The concerted action of phosphoinositide kinases and phosphoinositide phosphatases generates multiple polyphosphoinositides phosphorylated at the 3, 4 and 5 positions either alone or in combination. Although each of the phosphoinositide kinases can utilize multiple substrates in vitro, for the sake of simplicity, only those reactions which are likely to predominate in vivo are highlighted. The black solid arrows show potential routes of synthesis which have been demonstrated in vitro, whereas broken black arrows show dephosphorylation reactions. The red arrows highlight the physiologically relevant routes of synthesis and the enzymes responsible for the reactions, whereas the blue arrows show the in vivo dephosphorylation reactions and the relevant phosphatases. In some cases, the predominant routes of synthesis in vivo are still unclear; e.g. the type II PIP kinase can use both PtdIns-3-P and PtdIns-5-P as a substrate, but in vivo it is only predicated that the major reaction carried out for this enzyme is PtdIns-5-P to PtdIns-4,5-P_2. For additional detail, see text.

A unique insight into the regulation of PIP kinases came recently with the crystal structure of PIP kinase IIβ [12]. The structure revealed two identical subunits interacting at the N-terminus, and this generates an elongated, flat structure whose surface is covered with highly basic, positively charged residues. This provides an ideal surface for interaction with negatively charged phosphoinositides such as PtdIns-5-P. This immediately provides an explanation for the high-affinity interaction of phosphoinositide kinases with substrates; what remains is a structural explanation for the specificity of different kinases for distinct lipids which have either a 3, 4 or 5OH group available for phosphorylation. This will ultimately be resolved by solving the crystal or solution structures of other phosphoinositide kinases and in particular PI 3-Ks. An additional route of synthesis for PtdIns-4,5-P_2 generation is dephosphorylation of the PI 3-K lipid PtdIns-3,4,5-P_3 by 3OH phosphatases. As discussed below, one such phosphatase is the tumor-suppressor gene PTEN/MMAC1, but whether in vivo this pathway serves to significantly contribute to PtdIns-4,5-P_2 levels, or whether it is only a mechanism to remove the PtdIns-3,4,5-P_3 signal

remains to be established. For recent and comprehensive reviews on PIP kinases, see Hinchcliffe et al. [4] and Anderson et al. [5].

The simultaneous discovery of PtdIns-3-P and PtdIns-3,4,5-P_3 by different laboratories in the mid 1980's indicated that a new, agonist-stimulated signaling pathway exists which involves three novel phosphoinositides, PtdIns-3-P, PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 [13–15]. Work in the last decade has indicated that as with PtdIns-4-P and PtdIns-4,5-P_2, a family of PI 3-K exists which is responsible for the regulation of D3 phosphoinositide levels inside the cell. Three distinct PI 3-kinases families have been described, each with well-defined substrate specificities. Extensive characterization has been done on the class I PI 3-Ks, and it is these enzymes for which there exists considerable information on their regulation. For a recent comprehensive review on the PI 3-K enzyme family, see Wymann and Pirola [6]. Class Ia enzymes are typically activated by stimulation of cells with growth factors, hormones and other stimuli which operate through receptor and nonreceptor tyrosine kinases. Class Ia enzymes are composed of two subunits, a regulatory p85 protein (subtypes α, β and γ), and a catalytic p110 subunit (subtypes α, β and δ). Class Ia enzymes are ubiquitously expressed, and their mechanism of regulation by interaction with phosphotyrosine-containing sequences or receptors is completely understood. Similarly, these enzymes require a direct interaction with the small GTPase p21Ras for efficient catalytic function [16]. Class Ib PI 3-Ks are typically activated by ligands which use G-protein-coupled receptors to initiate signaling (e.g. *N*-formyl-Met-Leu-Phe stimulation of neutrophils and thrombin stimulation of platelets). Class Ib PI 3-Ks, also known as PI 3-Kγ, are composed of a catalytic p120 subunit and a p101 regulatory subunit, which unlike p85 has no SH2 or SH3 domains to serve as a receptor docking mechanism [17]. p101 appears to confer sensitivity to G$\beta\gamma$ subunits and may posses a PH domain which would allow for membrane localization. Class Ib enzymes also require binding to Ras-GTP for catalytic function. Important for their function in initiating downstream signaling is the fact that at least in vitro, class I enzymes can utilize all three substrates PtdIns, PtdIns-4-P and PtdIns-4,5-P_2 to generate PtdIns-3-P, PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3. In vivo, it is likely that class I enzymes serve as the major route for PtdIns-3,4,5-P_3 synthesis by phosphorylating PtdIns-4,5-P_2 [18] (fig. 1). In vitro, class I enzymes can also phosphorylate PtdIns-5-P to generate PtdIns-3,5-P_2, but in cells it is likely that the major route of synthesis is phosphorylation of PtdIns-3-P by a PI 5-kinase.

Class II PI 3-Ks have a more restricted substrate specificity and can only efficiently phosphorylate PtdIns and PtdIns-4-P to generate PtdIns-3-P and PtdIns-3,4-P_2. These enzymes are characterized by the presence of a C2 domain, and although this does appear to confer calcium sensitivity to these enzymes, it appears to be essential for catalytic function [19]. These PI 3-Ks, of which α, β and γ isoforms have been described and cloned, do not appear to have a regulatory subunit associated with them, and thus their mechanism of regulation remains unknown, though there are reports of tyrosine phosphorylation which could mediate a potential association with receptors [20]. Inside the cell, these enzymes are likely to be a primary source for the synthesis of PtdIns-3,4-P_2 (fig. 1). Thus elucidation of their regulation and role in signaling remains an important goal.

Finally, class III enzymes have a unique substrate specificity in that they are only capable of phosphorylating PtdIns to generate PtdIns-3-P (fig. 1). The first cloned member of this family was the yeast protein Vps34p [21]. Yeast cells expressing mutant forms of this protein have severe defects in vacuolar protein sorting, and these cells have no detectable levels of PtdIns-3-P [22, 23]. A large number of yeast, *Caenorhabditis elegans*, *Drosophila melanogaster* and mammalian homologues of Vps34p have now been identified and cloned [24]. In all of these organisms, class III enzymes appear to participate in vesicular sorting, endocytic and exocytic mechanisms. In yeasts, Vps34p is tightly associated with a serine-threonine protein kinase activity which was identified as Vps15p [22], and a functional human homologue (p150) has also been identified [25]. It is clear that in yeasts, Vps34p is the only route of synthesis for PtdIns-3-P. In higher eukaryotes, however, both class II and class I PI 3-Ks have the potential to generate this lipid. Although this needs more stringent clarification, it is likely that in mammalian cells the majority of PtdIns-3-P is generated by PtdIns 3-kinases.

Phosphoinositide phosphatases

The most information concerning phosphoinositide phosphatase function and regulation lies with the 5OH phosphatases, which specifically dephosphorylate phosphoinositides with a 5′ phosphate, such as PtdIns-5-P, PtdIns-3,5-P_2, PtdIns-4,5-P_2 and PtdIns-3,4,5-P_3. 5OH phosphatases have been classed into five distinct subfamilies, of which type I enzymes only dephosphorylate the soluble inositol phosphates. Class II enzymes, on the other hand, dephosphorylate both soluble inositol phosphates as well as phosphoinositides. An important member of this family is the product of the OCRL [oculocerebrorenal (Lowe)] syndrome [26]. Synaptojanin is a phosphatase also belonging to the class II enzymes, and appears to participate in synaptic vesicle trafficking [27]. As discussed above, the SHIP1 and SHIP2 phosphatases (class III enzymes) are important regulators of both PtdIns-3,4,5-P_3 as well as PtdIns-4,5-P_2, as these enzymes are capable of dephosphorylating both substrates

by removing the 5OH position (fig. 1). SHIP1 appears to be restricted to cells of hematopoietic origin and participates in cytokine-dependent signaling [28]. Targeted disruption of this SHIP1 results in myeloid proliferation, and death from infiltration of the lungs by myeloid cells [29]. SHIP2 has a more ubiquitous distribution and appears to participate in growth-factor-dependent signaling downstream of receptor-tyrosine kinases by coupling to the adapter molecule Grb2 as well as Shc [30]. Type IV phosphatases are poorly understood and appear to selectively dephosphorylate PtdIns-3,4,5-P_3.

4OH phosphoinositide phosphatases are also relatively poorly understood but appear to participate in PI 3-K signaling. These enzymes dephosphorylate PtdIns-3,4-P_2 to yield PtdIns-4-P, and thus are likely to downregulate or terminate signaling downstream of this lipid. Equally mysterious are the 3OH phosphatases, which specifically utilize PI 3-K lipids as substrates and also lead to a termination of PI 3-K signaling. An important member of this family which has received considerable attention recently is the PTEN/MMAC1 protein, which is the product of a tumor suppressor gene [31]. PTEN specifically removes the 3OH phosphate of PtdIns-3,4,5-P_3, resulting in the production of PtdIns-4,5-P_2 [32] (fig. 1). This particular protein has captured the attention of workers in the field as several mutations or deletions in this gene are found in a wide variety of human cancers (reviewed by Cantley and Neel [33]). Germ-line mutations in this gene are also responsible for the dominantly inherited disease Cowden syndrome, which is characterized by multiple hamartomas [33]. Similarly, PTEN is capable of suppressing the growth of glioma cells, and mutations in PTEN which abolish catalytic function lead to hyperphosphorylation of the PI 3-K effector Akt/PKB (see below) [34]. This has provided another link between PI 3-K, PtdIns-3,4,5-P_3, and cell proliferation and cancer. Clearly, other phosphoinositide phosphatases must exist which can specifically dephosphorylate phosphoinositides, providing a negative signal. Discovery and characterization of these proteins and their activities will no doubt add another layer of complexity of phosphoinositide phosphorylation and dephosphorylation reactions. For a recent review of phosphoinositide phosphatases, see Majerus et al. [35].

Phospholipases

None of the PI 3-K lipid products – PtdIns-3-P, PtdIns-3,4-P_2, PtdIns-3,4,5-P_3 – are substrates for any phospholipase type C (PLC) enzymes. However, a recent paper provided intriguing evidence that a family of novel phospholipase type D (PLD) enzymes exist in brain which convert both PtdIns-3-P and PtdIns-3,4,5-P_3 (but not PtdIns-4,5-P_2 or PtdIns-3,4-P_2) into soluble inositol phosphates Ins-3-P and Ins-3,4,5-P_3 [36]. It is presently unclear how relevant these PLD's are in hydrolyzing D3 phosphoinositides in vivo. PtdIns-4-P and PtdIns-4,5-P_2 are hydrolyzed by PLCs to yield additional important second messengers, Ins-1,4-P_2 (when PtdIns-4-P is the substrate) or Ins-1,4,5-P_3 (IP_3, when PtdIns-4,5-P_2 is the substrate) plus DAG. The latter is an important intracellular lipid second messenger which directly binds to and activates the protein kinase C (PKC) family of enzymes. As the structure-function relationships of the phosphoinositide-specific PLC family have been comprehensively reviewed recently [37], here we will only discuss the important aspects of PLC regulation as well as some key observation made in the last few years. Although three PLC subtypes exist (PLCδ, PLCγ and PLCβ, each with a unique mechanism of regulation), they all appear to share a common catalytic mechanism employing calcium as a cofactor [37]. Insight into this mechanism was obtained from crystallographic studies of the PLCδ-1 enzyme, which revealed an irregular α/β barrel [38, 39]. Resolution of the PLCδ-1 structure in complex with both calcium and IP_3 revealed residues critical for the interaction which are conserved among other PLC subtypes. In contrast, PLCδ subtypes diverge when one considers their mechanism of regulation. Up to four distinct PLCδ subtypes have been identified in mammalian cells, and although the mechanism of regulation is still under investigation, a novel class of GTP-binding protein termed Gh may be important for PLCδ regulation [40]. Unlike other PLCs, there is also evidence that increase in calcium concentration alone is sufficient to activate the enzyme [41].

Of particular importance to PLCδ function is the presence of a PH domain, and indeed, binding of both PtdIns-4,5-P_2 and the soluble head group IP_3 to the isolated PH domain of PLCδ-1 has been demonstrated [42, 43]. Although there is evidence that the binding of PtdIns-4,5-P_2 to the PLCδ PH domain alone can influence activity, in cells this is unlikely to be the major catalytic mechanism by which PLCδ is activated. The relative contribution of the PH domain, calcium, Ghα and other upstream regulators for the full and efficient activation of PLCδ clearly requires further work. Although PLCβ subtypes ($\beta1-\beta4$) also have a PH domain, curiously this does not appear to mediate PtdIns-4,5-P_2 binding, and so it may be responsible for other interactions, possibly with other proteins. PLCβ enzymes are regulated by binding to heterotrimeric GTP-binding proteins. It appears that both G$\beta\gamma$ as well as Gqα subunits are required for efficient PLCβ activation, presumably by interacting with distinct domains in the enzyme [37]. Finally, PLCγ subtypes (PLCγ-1 and PLCγ-2) are activated by virtually all polypeptide growth factors and other ligands which couple to receptor tyrosine kinases. The major mechanism of regulation of these enzymes involves tyrosine phosphorylation of the PLC at three key phosphorylation sites, and this is required for catalytic function [44].

PLCγ enzymes have two copies of an SH2 domain and one copy of an SH3 domain. The former are required for interaction with phosphotyrosine-containing sequences in receptor-tyrosine kinases such as the platelet-derived growth factor-receptor (PDGF-R). The SH3 domain has the potential of interacting with polyproline rich sequences such as that of dynamin, though this requires confirmation in vivo [44]. Particularly intriguing for PLCγ function is the finding that the SH2 domains of PLCγ can bind to PtdIns-3,4,5-P_3 in vitro, leading to activation of the PLC [45]. There is also a report of PtdIns-3,4,5-P_3-binding to the isolated PH domain of PLCγ [46]. These interactions may be physiologically relevant, as expression of activated PI 3-K mutants in vivo leads to a relocalization of PLCγ to the plasma membrane [45]. In addition, there are other convincing reports of cross-talk between the PI 3-K and PLCγ pathways [47], and PtdIns-3,4,5-P_3 may be an important regulator of PLCγ activity in cells. Other lipids such as arachidonic acid and phosphatidic acid have been shown to upregulate the activity of PLCγ, at least in vitro, but whether these operate in the absence of tyrosine phosphorylation is not known [44]. Thus PLCγ has the potential to be regulated by both protein-protein interaction (SH2 with P-Tyr and SH3 with dynamin), direct phosphorylation by Src and receptor tyrosine kinases, and lipid-protein interaction (PH/SH2 domain with PtdIns-3,4,5-P_3). This complexity underscores the importance of PLCγ signaling in normal and transformed cells, and the role of its products IP_3 and DAG in mediating cell proliferation, differentiation and cell motility.

Proteins and protein modules which bind to phosphoinositides

Having discussed the regulation of the metabolism of the various phosphoinositides by kinases, phosphatases and phospholipases, we will now turn our attention to the lipids themselves and the mechanism by which they initiate downstream signaling pathways. At this point it worth noting that although phosphoinositide synthesis remains the major mechanism of action of the PI kinases, some of the cellular processes attributed to these enzymes may actually be attributable to a separate activity. A large family of protein kinases exist which show homology with the catalytic domains of PI 3- and PI 4-kinases [48]. However, these kinases do not seem to posses intrinsic lipid kinase activity. Conversely, most of the phosphoinositide kinases do not appear to possess protein kinase activity, with one notable exception: the class I PI 3-K are dual-specificity kinases which phosphorylate proteins as well as phosphoinositides. Until recently, the only known substrate of this protein kinase activity was the p85 regulatory subunit of the PI 3-K itself, and this serves to inactivate the lipid kinase activity [49, 50]. However, recent work from Bondeva et al. has clearly demonstrated that a major function of the protein kinase activity of p85/p110 PI 3-K is to activate mitogen-activated protein kinase (MAPK) [51], though the direct substrate of the PI 3-K remains unclear. Thus it appears that at least in this system, the phosphoinositide kinase activity of PI 3-K is dispensable for MAPK activation. Whether other PI kinases also possess a physiologically relevant protein kinase activity remains to be established.

PtdIns-3-P, PtdIns-4-P and PtdIns-5-P

All three of the monophosphorylated lipids, PtdIns-3-P, PtdIns-4-P and PtdIns-5-P, are constitutively present in cells, and their levels do not appear to fluctuate in most cell types studied to date. Two notable exceptions to this rule are the finding that PtdIns-3-P levels do increase transiently in platelets stimulated with an antibody which mimics integrin function [52, 53]. Similarly, we have also found that in isolated platelets levels of PtdIns-4-P do increase upon stimulation with thrombin [54]. Whether these fluctuations are restricted to platelets or can also be found in other cells remains to be seen. Similarly, as PtdIns-5-P was discovered only in the last year, there is little information concerning the regulation of its synthesis in cells, so it is presently unclear whether its levels change upon cell stimulation. Until recently, the function of PtdIns-3-P was unclear. The finding that the PtdIns kinase which is responsible for its synthesis in yeasts, the Vps34 protein, was involved in vacuolar protein sorting mechanisms provided the first clue that this lipid might somehow regulate vesicle trafficking. Recent work has uncovered a function for PtdIns-3-P in trafficking events. A 70-residue protein sequence called the FYVE finger domain has been found to interact specifically with PtdIns-3-P and not other phosphoinositides [55–57]. This domain is similar to RING zinc-finger domains [55], and several FYVE-containing proteins have been shown to interact specifically with PtdIns-3-P, such as the early endosome antigen-1 (EEA1) [56–58] and the yeast proteins Vac1p, Vps27p , Fab1p and Pib1p [56, 58]. Another PtdIns-3-P-interacting protein is the adapter protein-2 (AP-2) which is involved in assembling clathrin coats at the membrane [59]. Although AP-2 can also bind weakly to PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 [60], the relatively higher specificity of PtdIns-3-P for this protein suggests that this is a physiologically relevant interaction. As discussed below, these various proteins link PtdIns 3-kinases and PtdIns-3-P to vesicle trafficking (for a recent review on FYVE-containing proteins, see Wurmser et al. [24]).

Little or no information exists concerning PtdIns-4-P-binding proteins. Indeed, it has long been presumed that

the sole function of this lipid is to act as a substrate for PIP kinases to generate PtdIns-4,5-P_2, as well as a substrate for PI 3-Ks to produce PtdIns-3,4-P_2. However, several studies have demonstrated that the large number of currently identified PtdIns-4,5-P_2-binding proteins also have the potential to interact with PtdIns-4-P, suggesting that this lipid may have a more general function in the cell that had previously been assumed [37]. Similarly, only in the last year have we become aware of the presence of PtdIns-5-P in cells, and it is not known whether there are proteins which directly interact with this lipid, or whether it only serves as a substrate for the production of PtdIns-4,5-P_2 or PtdIns-3,5-P_2. PtdIns-5-P constitutes only 2% of the total cellular PIP in the cell [8]. As PtdIns-3-P is present at about the same levels (5%), this does not discount the fact that PtdIns-5-P-binding proteins exist.

PtdIns-3,4-P_2 and PtdIns-3,5-P_2

Although it has always been presumed that PtdIns-3,4-P_2 is merely a breakdown product of PtdIns-3,4,5-P_3 hydrolysis and thus not function as a true second messenger per se, recent work has demonstrated that it can directly interact with several proteins and affect their activities and/or localization. The best-studied PtdIns-3,4-P_2 effector is the Akt/PKB protein kinase, which as discussed below is also a target for PtdIns-3,4,5-P_3. The PH domain of Akt/PKB binds with high affinity and specificity to phosphoinositides in the following order of preference: PtdIns-3,4-P_2 > PtdIns-3,4,5-P_3 ⩾ PtdIns-4,5-P_2 [61, 62]. Binding requires the conserved Arg25 residue in the PH domain of Akt/PKB, and mutation of this site abolishes both PtdIns-3,4-P_2/PtdIns-3,4,5-P_3 binding as well as mitogen responsiveness [61, 63]. Binding of PtdIns-3,4-P_2 to Akt/PKB induces a small (3–5 fold) but reproducible activation of the enzyme [61, 62, 64], but as discussed below, full and efficient activation of Akt/PKB requires phosphorylation at the activation loop Thr308. This is carried out by the recently discovered PDK-1 (phosphoinositide-dependent kinase 1) enzyme [65, 66]. PDK-1 itself also binds with high affinity to PtdIns-3,4-P_2, although it seems to have a higher affinity for PtdIns-3,4,5-P_3 in vitro [67, 68]. At the moment, it is somewhat unclear which of the two lipids, PtdIns-3,4-P_2 and/or PtdIns-3,4,5-P_3, is required for activation of Akt/PKB in vivo. Evidence for a requirement for PtdIns-3,4-P_2 comes from studies in platelets in which Akt/PKB activation correlates with PtdIns-3,4-P_2 and not PtdIns-3,4,5-P_3 accumulation [52, 61, 69]. Ultimately, a complete explanation for the requirement of PtdIns-3,4-P_2 and/or PtdIns-3,4,5-P_3, or both, for Akt/PKB activation will have to come from structural studies of the enzyme bound to these lipids. Certain novel PKCs such as PKCδ, PKCε and PKCξ are also activated directly in vitro by PtdIns-3,4-P_2 [70–73], but as discussed below, this activation is likely to require the concerted action of both lipids as well as PDK-1. What has yet to be demonstrated is the binding site of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 on these PKCs. As these enzymes do not have a PH domain, it is likely that a distinct region in the regulatory domain of these enzymes is responsible for the lipid-protein interaction. Thus, it is clear that PtdIns-3,4-P_2 has the potential for acting as a true lipid second messenger, and the identification of additional proteins which interact with PtdIns-3,4-P_2 will reinforce this notion.

Although no putative targets of PtdIns-3,5-P_2 have been discovered to date, it is likely that this is only a matter of time. The levels of PtdIns-3,5-P_2 have been shown to change dramatically in a variety of cell systems. Hyperosmotic shock of yeast cells results in rapid increase in PtdIns-3,5-P_2 levels, whereas in mammalian cells the same treatment results in a slight decrease, although an increase has been seen following hypoosmotic shock [74]. Again, PH domains are good candidates for PtdIns-3,5-P_2 binding, and future studies on phosphoinositide-binding specificity will no doubt take this into account.

PtdIns-4,5-P_2

As the presence of PtdIns-4,5-P_2 in cells has been known for several decades, it is not surprising that many proteins which directly interact with this lipid have been discovered. As discussed here, many of these proteins are intimately associated with the regulation of the actin cytoskeleton. However, PtdIns-4,5-P_2 has many other activities besides regulating actin remodeling, and in addition to serving as a substrate for phosphoinositide kinases, phosphatases and phospholipases, it also affects the activities of several enzymes.

PtdIns-4,5-P_2 has been shown to promote actin polymerization directly by interacting with profilin, an actin-binding protein (fig. 2). Profilin sequesters actin monomers, thus preventing polymerization of filaments, and PtdIns-4,5-P_2 disrupts this association [75, 76]. It is, however, worth noting that profilin has also been shown to interact with both PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 [77], and given the evidence for a role for PI 3-K in cytoskeletal rearrangement, such interactions cannot be discounted as physiologically irrelevant. Actin filaments are also prevented from spontaneously polymerizing by being "capped" at the barbed by ends by capping proteins such as CapZ and related proteins, and again, PtdIns-4,5-P_2 has been shown to interfere with this interaction leading to polymerization [78]. Indeed, permeabilized platelets show an increase in actin polymerization and the number of free barbed ends when PtdIns-4,5-P_2 is added, though similar results are obtained with both PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 [54, 79]. In this model

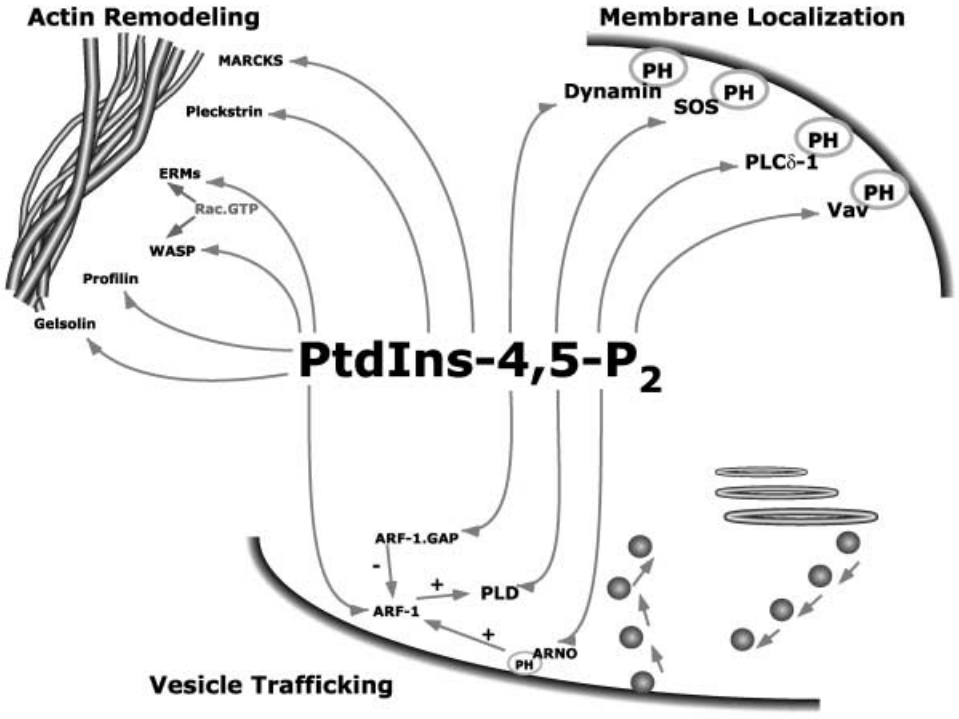

Figure 2. The role of PtdIns-4,5-P_2 in signaling. A large number of PtdIns-4,5-P_2 effectors regulate actin cytoskeleton remodeling, membrane localization and vesicle trafficking processes. Interaction of PtdIns-4,5-P_2 with actin capping and severing proteins such as profilin and gelsolin (as well as many others) leads to actin polymerization. The small GTPase Rac acts in synergy with PtdIns-4,5-P_2 to regulate ERM (ezrin/radixin/moesin) and WASP proteins, and the PKC substrates MARCKS and pleckstrin have also been shown to regulate the actin cytoskeleton in a PtdIns-4,5-P_2-dependent manner. High affinity binding of PtdIns-4,5-P_2 to a number of proteins causes them to relocalize to the plasma membrane, and this is typically mediated by the PH domain of phospholipases (e.g. PLCδ-1) and exchange factors of GTPases (e.g. Sos, Vav). In the case of Vav, PtdIns-4,5-P_2 binding inhibits exchange activity, whereas PtdIns-3,4,5-P_3 binding stimulates activity towards the Rac GTPase (see fig. 3). Finally, PtdIns-4,5-P_2 is a critical regulatory component of intracellular vesicle trafficking. In particular, PtdIns-4,5-P_2 is an essential cofactor for PLD, which regulates intracellular membrane traffic. In addition, ARF-1, which acts in concert with PtdIns-4,5-P_2 to activate PLD, is also regulated by PtdIns-4,5-P_2 binding. The exchange factor for ARF-1, ARNO, also binds to PtdIns-4,5-P_2 through its PH domain. Finally, inactivation of ARF-1. GTP is mediated by its ARF-GAP, and this reaction also requires PtdIns-4,5-P_2.

system, the GTPase Rac also plays a central role, as it interacts in the GTP-bound form with the PIP kinase responsible for PtdIns-4,5-P_2 synthesis [54] (fig. 2). Other important regulators of the actin cytoskeleton such as gelsolin, cofilin, gCAP39, α-actinin, filamin and vinculin also interact with PtdIns-4,5-P_2 (reviewed by Janmey [80]). Another link between PtdIns-4,5-P_2 and the cytoskeleton is ERM (ezrin/radixin/moesin) family members, which interact with both surface glycoproteins (e.g. CD44) and actin filaments [81]. This association is enhanced by PtdIns-4,5-P_2, and as ERM proteins are effectors of Rho family GTPases [82], this links PtdIns-4,5-P_2, Rac and ERM with the actin cytoskeleton (fig. 2). Similarly, the PH domain-containing protein WASP (Wiskott-Aldrich syndrome protein), defective in patients with the disease, binds PtdIns-4,5-P_2, and is an effector of Cdc42 and also binds to the actin cytoskeleton [83]. Finally, the PKC substrates pleckstrin (the prototype of the PH domain) and MARCKS (myristoylated alanine-rich C-kinase substrate) have also been shown to interact with PtdIns-4,5-P_2 and promote association with the cytoskeleton [84, 85] (fig. 2). Thus there is a wealth of in vitro evidence which makes a good case for the central role of PtdIns-4,5-P_2 in actin remodeling. In vivo evidence has been less forthcoming, but studies in yeast have corroborated this hypothesis; yeast cells lacking Mss4p (a PIP kinase homologue) lack the ability to form actin filaments and to correctly assemble their cytoskeleton, effects which can be rescued with expression of the human PIP kinase [86].

Several other proteins interact directly with PtdIns-4,5-P_2, and most of these have PH domains. Particularly intriguing are proteins which regulate intracellular vesicle trafficking. PtdIns-4,5-P_2 is a cofactor for PLD activity [87]. Similarly, PtdIns-4,5-P_2 stimulates association of GTP with ARF-1, a known activator of PLD [88]. An exchange factor for ARF, named ARNO, was also described recently and was shown to interact with PtdIns-4,5-P_2 through its PH domain serving as a mechanism for membrane localization [89, 90]. Thus both ARF-GTP and PtdIns-4,5-P_2 act in concert to potently activate PLD [91] (fig. 2). In addition, PtdIns-4,5-P_2 stimulates inactivation

of ARF-GTP, as ARF-GAP is also activated by PtdIns-4,5-P_2, causing Arf-1 to revert back to the inactive GDP-bound state [92].

The list of PtdIns-4,5-P_2-binding proteins does not end here. Several others, some with PH domains, have also been shown to interact with PtdIns-4,5-P_2, but what is lacking is a sense of the physiological relevance of this interaction. PtdIns-4,5-P_2 can disrupt the association with Rac and its RhoGDI [93], lead to GDP/GTP exchange on Cdc42 [94], and bind to the exchange factor Sos [95–98] (fig. 2). In the case of Sos, though there is physiological evidence for the interaction, it is far from clear what the role of PtdIns-4,5-P_2 is. Although the isolated PH domain of Sos can bind to PtdIns-4,5-P_2 in vitro, mutations which abrogate this binding do not affect membrane localization in vivo, suggesting that other ligands may be relevant for the PH domain [98]. Also noteworthy here is the finding by one group of a higher-affinity interaction of PtdIns-3,4,5-P_3 over PtdIns-4,5-P_2 for the PH domain of Sos [95]. The GTPase dynamin also binds through its PH domain to PtdIns-4,5-P_2, and this appears to stimulate its GTPase activity [99–101]. In summary, many proteins exist which are direct effectors of PtdIns-4,5-P_2 in the cell. In most cases, this interaction is mediated though a PH domain, and indeed, a structural explanation for some of these exists (e.g. PLCδ-1). On the other hand, PtdIns-4,5-P_2 binding domains or sequences in other proteins such as the actin-capping and severing proteins linked to actin remodeling remain somewhat obscure. Clearly establishing a direct correlation between PtdIns-4,5-P_2 protein-binding in vitro and equivalent function in vivo remains a challenge to workers in the field.

PtdIns-3,4,5-P_3

The cumulative efforts of a number of laboratories working on PI 3-K signaling has resulted in the discovery of a large number of proteins which have been shown to directly interact with PtdIns-3,4,5-P_3 thus transducing the PI 3-K signal inside the cell. PtdIns-3,4,5-P_3 is nominally absent in all serum-starved and unstimulated cells, and it accumulates rapidly upon stimulation with a variety of extracellular ligands, including growth factors and hormones. Local accumulation of PtdIns-3,4,5-P_3 at the site of PI 3-K activation results in the recruitment of proteins which directly interact with the lipid. One of the best studied is the Akt/PKB protein kinase, the cellular homologue of the viral oncogene *v-Akt* [63]. As already discussed, PtdIns-3,4-P_2 can weakly activate the protein kinase activity of Akt/PKB *in vitro*, but the primary role of PtdIns-3,4-P_2/PtdIns-3,4,5-P_3 binding to the PH domain of Akt/PKB is to induce a conformational change in the protein which exposes its activation loop threonine (Thr308). A tremendous boost in the field was the discovery of the PDK-1 enzyme which directly phosphorylates Thr308 in Akt/PKB, leading to a potent activation [65,66] (fig. 3). PDK-1 is related to both Akt/PKB as well as the extended PKC superfamily [67, 102], and it has a PH domain at the C-terminus. Interestingly, PtdIns-3,4,5-P_3 binding to the PDK-1 PH domain does not appear to directly stimulate its protein kinase activity [102]. Rather, this binding serves to relocalize the enzyme from the cytosol to the plasma membrane [103].

Equally important for full Akt/PKB regulation is phosphorylation of the C-terminal hydrophobic site Ser473. It has been shown that phosphorylation of this site is also PI 3-K, and thus presumably PtdIns-3,4,5-P_3 dependent, and thus the putative enzyme responsible for carrying out the reaction termed PDK-2 [104]. Although there is one report that an integrin-linked kinase (ILK) is capable of phosphorylating Ser473 [105], it is not clear this is physiologically relevant, and thus the search for PDK-2 continues. However a very recent report indicates that PDK-2 may in fact not exist. The group of Alessi has shown that PDK-1 can acquire PDK-2-like activity (i.e. phosphorylate Ser473) in the presence of a peptide of the PKC-related kinase PRK-2, which apparently shifts the substrate specificity of PDK-1 from Thr308 to Ser473 [106]. Whether this represents a physiologically relevant mechanism remains to be seen, but clearly this is an interesting possibility given that many other kinases in this family have a similar hydrophobic-directed site in their C-terminus. Several PKC family members, including PKCδ, PKCε, PKCη, PKCζ and PKCλ, have been shown to be directly activated by both PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 in vitro. Similarly, in vivo studies have clearly demonstrated that both PKCζ, PKCλ and PKCε are activated downstream of PI 3-K in stimulated cells [72, 107]. Recent work from several laboratories has shown that maximal activation of PKCδ and PKCζ requires phosphorylation of their activation loop threonines by PDK-1 in vitro [108–110] (fig. 3). Maximal phosphorylation and activation of these PKCs by PDK-1 requires the presence of PtdIns-3,4,5-P_3. Thus a similar mechanism of regulation by both PDK-1 and PtdIns-3,4,5-P_3 has been proposed for these PKCs.

The site of PtdIns-3,4,5-P_3 interaction on PKCs remains undescribed. Although for novel (e.g. PKCδ) and atypical (e.g., PKCζ) PKCs phosphorylation induced by PDK-1 clearly leads to activation of the protein kinase, for conventional PKCs (e.g. PKCα and PKCβII) this does not appear to be the case. PDK-1 does indeed phosphorylate the activation loop threonine of these PKCs, but this does not activate the PKC; rather, it allows for subsequent autophosphorylations in the catalytic domain of the enzymes [111]. Several other protein kinases have been shown to be regulated by PDK-1, but in a PtdIns-3,4,5-P_3-independent manner (fig. 3). p70S6-kinase, an enzyme involved in translational regulation of a number of important messenger RNAs (mRNAs), is also phosphor-

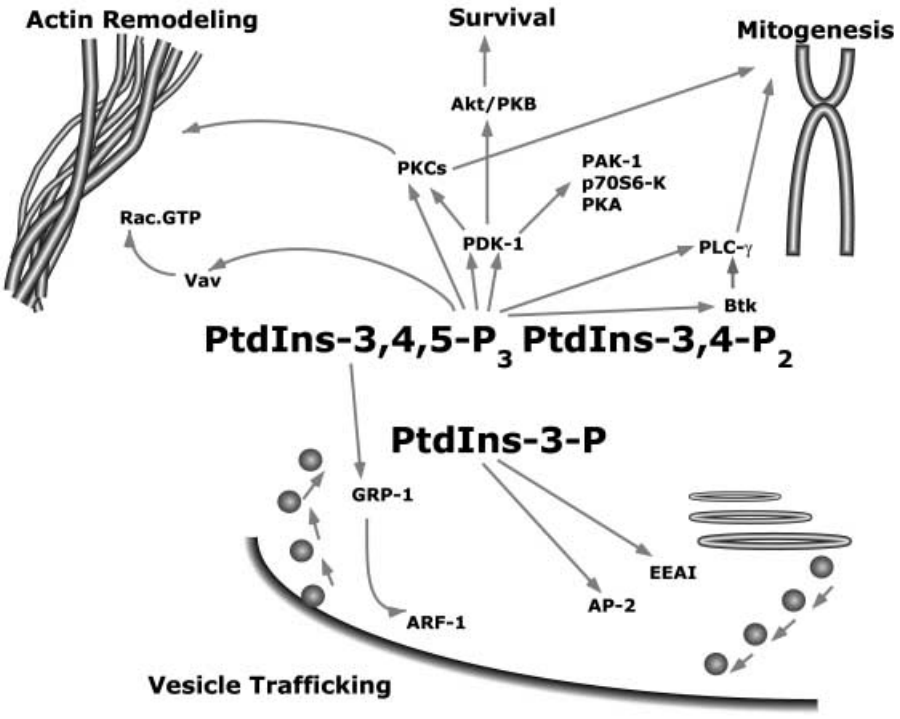

Figure 3. The role of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 in signaling. A large number of PtdIns-3,4-P_2- and PtdIns-3,4,5-P_3-binding proteins exist in the cell whose activities or cellular location are altered by lipid binding. These interactions can have significant effects on cell fate. Remodeling of the actin cytoskeleton is mediated by PtdIns-3,4,5-P_3 binding and activation of the exchange factor for Rac, the Vav protein. There is also the indication that certain PKC family members can influence the cytoskeleton. Cell survival in response to pro-apoptotic stimuli is mediated by the PtdIns-3,4,5-P_3-PDK-1-Akt/PKB pathway. Similarly, both PKCs and PLCγ have been implicated in cell cycle progression and mitogenesis in a PI 3-K-dependent manner, and both of these enzymes are regulated by PtdIns-3,4,5-P_3 . PI 3-K can influence vesicle trafficking by either PtdIns-3,4,5-P_3 binding to the GRP-1 protein, which can affect ARF-1 activity, or by PtdIns-3-P, which specifically interacts with FYVE-containing proteins such as EEA1, as well as the AP-2 protein, which mediates clathrin coat assembly at the membrane.

ylated by PDK-1, leading to activation, but this reaction does not require PtdIns-3,4,5-P_3 [112, 113]. PKA, the prototype of this family, is also a substrate for PDK-1 in vitro, but it is unclear whether in vivo this is the relevant kinase for PKA [114]. Are there other PDK-1 targets in the cell? The answer is almost certainly yes. Unpublished results from our laboratory indicate that the p21-activated kinase PAK-1 is phosphorylated by PDK-1 in vitro and in cells, and indeed, there is evidence that PAK-1 is activated by PI 3-K-dependent mechanisms in the cell [115].

A number of additional protein kinases have been shown to be PI 3-K and PtdIns-3,4,5-P_3 targets. The C-Jun N-terminal kinase (JNK) is activated by PI 3-K [116], but this appears to be a distal effector of PtdIns-3,4,5-P_3, which is presumably acting through the GTPase Rac (see below). Bruton's tyrosine kinase (Btk) is a PH domain containing tyrosine kinase which is involved in B lymphocyte development and is directly activated by the synergistic action of PtdIns-3,4,5-P_3 binding and phosphorylation by the Src tyrosine kinase [117, 118]. Importantly, mutations in the Btk PH domain which give rise to X-linked immunodeficiency in mice also abolish PtdIns-3,4,5-P_3 binding [119]. A series of elegant in vivo experiments have recently shown a relocalization of the Btk PH domain from the cytosol to the membrane in a PI 3-K-dependent manner, consistent with a role for PtdIns-3,4,5-P_3 in Btk regulation [120]. Finally, targeted disruption of the p85 PI 3-K regulatory subunit in mice results in defects in B cell development and proliferation, reminiscent of the phenotype of Btk-deficient mice [121–123].

Several of the small GTPases of the Ras/Rho superfamily have been shown to be activated downstream of PI 3-K. Rac was the first of these to be identified as a PI 3-K target, as GTP/GDP exchange on Rac requires efficient PI 3-K activity [124]. An attractive model for the regulation of Rac has been proposed in which the binding of PtdIns-3,4,5-P_3 to the Rac guanine nucleotide exchange factor Vav stimulates exchange activity towards Rac, RhoA and Cdc42 [125] (fig. 3). Interestingly, PtdIns-4,5-P_2 binding to Vav appears to inhibit its exchange activity. A more recent report has added another level of complexity to this model, as PtdIns-3,4,5-P_3 was reported to bind directly to Rac1 but not RhoA or Cdc42, stimulating GDP dissociation from Rac [126] To further complicate matters, although most studies have placed PI 3-K and PtdIns-3,4,5-P_3 upstream of Rac and its exchange factors,

there are reports placing Rac downstream of PI 3-K [127]. Whether this depends on cell type or stimulus used in unclear, but this is somewhat reminiscent of the confusion over the regulation of PI 3-K by Ras. There are reports of Ras being both upstream or downstream of PI 3-K [16, 128], although here it is clear that the overwhelming body of evidence places Ras as a necessary upstream regulator of PI 3-K. A number of other small GTP exchange factors have also been shown to be effector of PtdIns-3,4,5-P_3. The protein Grp1 (general receptor for phosphoinositides, also known as cytohesin) was cloned for its ability to interact in vitro with PtdIns-3,4,5-P_3 [129]. Grp1 has a PH domain which has high selectivity for PtdIns-3,4,5-P_3 and not other phosphoinositides, but in addition has a Sec7 domain which functions as an exchange factor for the small G proteins ARF1 and ARF5, and exchange activity is promoted by PtdIns-3,4,5-P_3 [129, 130] (fig. 3). Centaurin-α was also cloned as a PtdIns-3,4,5-P_3-binding protein and shows homology to ARF-GTPase activating proteins [131]. As discussed below, these proteins most likely play a role in vesicular trafficking.

Finally, phospholipases have been shown to interact with PtdIns-3,4,5-P_3, in particular PLCγ. Rameh et al. originally showed that PtdIns-3,4,5-P_3 can directly bind to the SH2 domains of a number of signaling molecules, particularly Src and p85 PI 3-K [132]. Similarly, the SH2 domains of PLCγ bind to PtdIns-3,4,5-P_3, and this enhances its phospholipase activity in vitro [45]. Consistent with this observation is the finding that inhibition of PtdIns-3,4,5-P_3 synthesis in cells with wortmannin leads to a reduction of PDGF-stimulated IP_3 production, suggesting that PtdIns-3,4,5-P_3 is a positive regulator of PLCγ in vivo [133]. These data are further complicated by the finding that the PH domain of PLCγ can also bind to PtdIns-3,4,5-P_3 [46]. Irrespective of the precise mechanism, these data provide compelling evidence for considerable cross-talk between PLCγ and PI 3-K signaling at the level of PtdIns-3,4,5-P_3 production.

Thus, the list of PtdIns-3,4,5-P_3-interacting proteins is growing, and it is likely that many other proteins which interact with high affinity and specificity with PtdIns-3,4,5-P_3 await discovery, underscoring the importance of this lipid as a second messenger. With the advent of PtdIns-3,4,5-P_3-affinity columns as well as synthetic analogues, it is likely that this process of discovery will be a rapid one. Indeed, a novel PtdIns-3,4,5-P_3-binding protein (PtdIns-3,4,5-P_3-BP) with no known function has been discovered [134]. Interestingly, PtdIns-3,4,5-P_3-BP is localized in the nucleus, providing one of the first pieces of evidence that PI 3-K can function in the nucleus, although the presence of a nuclear phosphoinositide cycle has long been known [135].

Signaling pathways regulated by phosphoinositides

Cell growth and proliferation

The role of PI 3-K in mediating mitogenesis and transformation has long been known. In fact PI 3-K was originally discovered as a phosphoinositide kinase activity which was associated with the viral oncoproteins v-Src, v-Ros and polyomavirus middle T antigen, and mutants of middle T which were unable to complex with PI 3-K were also defective in cell transformation [136]. Since these initial discoveries, considerable effort has gone into understanding the mechanisms by which PI 3-K mediates mitogenesis and cell transformation. Mutants of the PDGF-R which are not capable of recruiting PI 3-K (as well as PLCγ) to the activated receptor fail to drive mitogenesis in the presence of PDGF [137] (fig. 3). Similarly, microinjection studies using inhibitory antibodies against the PI 3-K p110 and p85 subunits indicated that PI 3-K is necessary for cell cycle progression from G1 to S [138, 139]. S phase entry in response to mitogens has also been shown to be inhibited by wortmannin in several cell types. Conversely, constitutive activation of PI 3-K in a variety of cell types results in stimulation of DNA synthesis [140]. Although these various studies have provided compelling evidence that PI 3-K is required for efficient G1 progression and DNA synthesis, a recent study from the Kazlauskas laboratory has provided additional intrigue. Jones et al. have shown that PDGF stimulation of cells results in two peaks of PI 3-K activity as judged by PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 production, an early one peaking a 15–30 min, and an additional previously unknown peak of similar or greater magnitude at 3–5 h post-stimulation [141]. The surprising finding was that the first peak was completely dispensable for mitogenesis and cell cycle progression, whereas the second peak was absolutely necessary. Hence, one might deduce that the PI 3-K effector molecules responsible for transducing the mitogenic signal might be activated at these later times.

What might these molecules be? There is evidence linking both Akt/PKB as well as atypical PKCs such as PKCζ to mitogenesis [142, 143]. In the case of PKCζ, it is likely that this occurs by activation of the MAPK pathway. Other targets of PI 3-K include the cyclin D1 protein, which is upregulated during the late stages of G1 [144]. Recently, a number of studies have supported the idea that PI 3-K and its lipid products are necessary to induce cell transformation. A natural oncogenic variant of the PI 3-K catalytic subunit has been isolated from a chicken retrovirus (ASV16) that causes hemangiosarcomas and named *v-p3k* [145]. This oncogene causes elevation of both PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 levels as well as Akt/PKB activity in infected cells. A different oncogenic variant of PI 3-K was also described as a truncated version of the regulatory p85 subunit [146]. Finally, a com-

pelling case for PI 3-K-mediated cell transformation came from the discovery that the PTEN/MMAC1 tumor suppressor gene is a PtdIns-3,4,5-P_3 phosphatase. This gene is either deleted or muta-ted in a wide range of human cancers, and is able to suppress the growth of glioma cells in culture [33]. A likely mechanism for PTEN action is that in PTEN-deficient cells Akt/PKB activity is constitutive because of the elevated levels of PtdIns-3,4,5-P_3. As Akt/PKB mediates cell survival pathways, cells would be resistant to pro-apoptotic stimuli. Consistent with this hypothesis is the finding that transfection of PTEN-deficient cells with activated Akt/PKB reverses the PTEN phenotype [34]. It is also possible that other PI 3-K and PtdIns-3,4,5-P_3 effector molecules mediate the tumoriogenic potential of PTEN. Thus, it is becoming increasingly clear that PI 3-K is a major mediator of cell growth and transformation.

The Actin Cytoskeleton and Cell Motility

As discussed above, there is considerable in vitro evidence implicating PtdIns-4,5-P_2 as a major regulator of the actin cytoskeleton. In vivo evidence has been less forthcoming, but studies in yeast have corroborated this hypothesis; yeast cells lacking Mss4p (a PIP kinase homologue) lack the ability to form actin filaments and to correctly assemble their cytoskeleton, effects which can be rescued with expression of the human PIP kinase [86]. Mammalian cell studies have shown that overexpression of PIP kinases which are responsible for PtdIns-4,5-P_2 synthesis leads to an increase in actin polymerization in cells [147], whereas expression of SHIP which dephosphorylates PtdIns-4,5-P_2 has the reverse effect [148]. However, it is equally clear that PI 3-K lipid products, particularly PtdIns-3,4,5-P_3, are also required for remodeling of the actin cytoskeleton. In PDGF-stimulated fibroblasts, PI 3-K is essential for mediating cytoskeletal rearrangements [124, 149], and it is likely that in this and other cell types, a major PI 3-K effector leading to actin remodeling is the small GTPase Rac and its exchange factor Vav [150] (fig. 3). Microinjection studies have also shown that Ras mediates efficient membrane ruffling by the PI 3-K-Rac pathway in vivo [151]. Similarly, using a *Xenopus* oocyte cell-free system, Ma and et al. showed that both PtdIns-4,5-P_2 and PtdIns-3,4,5-P_3 are capable of stimulating actin assembly, but only in the presence of Cdc42 [152]. Similarly, although PtdIns-4,5-P_2 is able to induce uncapping of actin filaments in isolated platelets, both PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 have similar effects [79]. In addition, PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 are able to stimulate cell motility when added exogenously to cells [153]. Thus, it is becoming increasingly clear that PtdIns-4,5-P_2, PtdIns-3,4,5-P_3 and the small GTPases Rac and Cdc42 are all critical regulators of the actin cytoskeleton.

Cell Death

The regulation of the Akt/PKB protein kinase by PtdIns-3,4,5-P_3 and PDK-1 has received considerable attention recently because of the role played by Akt/PKB as a cell survival factor (fig. 3). The pro-apoptotic BAD protein is a direct substrate for Akt/PKB in vivo [154]. A model for the anti-apoptotic function of Akt/PKB has been proposed in which Akt/PKB-mediated phosphorylation of BAD results in its dissociation from Bcl-XL [154]. This then results in association of phosphorylated BAD with 14-3-3 proteins, preventing further association with Bcl-XL, thus preventing apoptosis [154]. As BAD has a restricted tissue distribution and yet Akt/PKB is capable of mediating survival even in cells lacking BAD, the challenge has been to describe novel targets of Akt/PKB which could fulfil this function. One such target was recently discovered, the FKHRL1 forkhead transcription factor which when phosphorylated by Akt/PKB again leads to association with 14-3-3 proteins and retention of the factor in cytoplasm [155]. In the dephosphorylated state, FKHRL1 likely leads to apoptosis by inducing the expression of genes which are critical for apoptosis, such as the Fas ligand. It remains to be seen whether additional Akt/PKB targets are also necessary for mediating anti-apoptotic signals.

Vesicular Sorting and Trafficking

There is considerable evidence linking both PtdIns-4,5-P_2 and PtdIns-3,4,5-P_3, as well as other phosphoinositides to exocytosis, endocytosis and vesicle trafficking. PIP-kinase and its product, PtdIns-4,5-P_2, have been shown to be essential for calcium-dependent exocytosis of catecholamine in PC12 cells [156]. Anti-PIP-kinase or anti-PtdIns-4,5-P_2 antibodies block calcium-dependent secretion, and thus PtdIns-4,5-P_2 plays a major role in exocytosis, at least in this system. Similarly, PtdIns-4,5-P_2 has been implicated in the regulation of endocytosis (fig. 2). Type Ib PIP kinase is required for efficient endocytosis of the human colony-stimulating factor-1 receptor (CSF-1R), and a truncated PIP kinase is able to prolong the presence of the receptor at the plasma membrane [157]. Although not critically demonstrated, PtdIns-4,5-P_2 is postulated to play a major role in this endocytosis. Critical in vivo evidence for this model has come from a study of late and early endocytic coated vesicle formation both of which are dependent on PtdIns-4,5-P_2 [158]. This is consistent with the fact that the AP-2 protein and the GTPase dynamin, both PtdIns-4,5-P_2-binding proteins, are necessary to drive coated vesicle formation. It is also worth noting that D3 phosphoinositides may be components of the endocytic machinery, as mutations in the PDGF-R which impair association with PI 3-K also interfere with the trafficking of this receptor to the lysosome [159]. Also noteworthy is the regulation of PLD by

PtdIns-4,5-P_2. PLC has been implicated in both trafficking and secretion, and its product, phosphatidic acid (PA), is a potent activator of PIP kinases. A hypothesis was therefore suggested in which a postive amplification loop involving PLD and PIP kinases would result in the local generation of both PtdIns-4,5-P_2 and PA at specific cell locations [91].

In addition to receptor-mediated endocytosis, D3 phosphoinositides, particularly PtdIns-3-P and PtdIns-3,5-P_2, have recently been shown to play a critical role in vesicle trafficking. As discussed above, the yeast Vps34p protein encodes a PtdIns 3-kinase which synthesizes PtdIns-3-P in yeasts, and mammalian homologues of Vps34p have also been isolated. Mutants of this Vps34p disrupt sorting of proteins to the vacuole. With the discovery of FYVE-motif-containing proteins as distinct PtdIns-3-P effector molecules, the function of both Vps34p and PtdIns-3-P is becoming clearer. The EEA1 protein interacts with PtdIns-3-P, and this is responsible for mediating correct endosomal association of the protein. Thus, a model has been proposed in which effectors such as EEA1 with FYVE motifs are PtdIns-3-P effectors and regulate anterograde Golgi-to-vacuole trafficking [56] (fig. 3). In the vacuole, the PtdIns-3-P signal would be terminated by the action of hydrolases. The recent finding that the yeast protein Fab1p is a PtdIns-3-P 5-kinase has added a new layer to this model. Fab1p is essential for maintaining normal vacuole morphology, and cells expressing a mutant Fab1p show no detectable levels of PtdIns-3,5-P_2 and a dramatic increase in vacuole size [160]. Thus, in yeasts PtdIns-3,5-P_2 is postulated to be generated by the action of Fab1p on PtdIns-3-P to produce PtdIns-3,5-P_2. This latter lipid is predicted to maintain vacuole membrane recycling and turnover. What remains to be described is effector molecules of Fab1p and PtdIns-3,5-P_2, which are responsible for mediating these events (for a review on yeast vacuolar/lysosomal phosphoinositide signaling, see Wurmser et al. [24]). In addition, as the yeast phosphoinositide kinases as well as their products have their equivalent mammalian counterparts, it will not be surprising to find similar function for these pathways in the higher eukaryotes.

Insulin and Glucose Transport

Finally, we will briefly discuss the role of the PI 3-K pathway in mediating insulin-dependent glucose transport. Of all the phosphoinositide kinases, PI 3-K is the one which has emerged as a critically important mediator of many of the physiological actions of insulin. Although PI 3-K has been demonstrated to mediate several of the physiological actions of insulin (e.g. Akt/PKB phosphorylates both GSK3 and PFK-2, providing a link between PI 3-K and PtdIns-3,4,5-P_3 to gluconeogenesis and glycolysis), here we will restrict the discussion to glucose transport, for which there exists considerable, if conflicting, information. For a more detailed review on the role of PI 3-K in insulin signaling, see Shepherd et al. [161]. One of the major physiological actions of insulin is to regulate the level of blood glucose, and a key step in this process is the ability of insulin to stimulate the translocation of GLUT4 transporters to the cell surface, thus accelerating the rate of glucose uptake into target tissues. One of the first pieces of evidence that PI 3-K was necessary for this event was the finding that wortmannin could block GLUT4 translocation in adipocytes [162]. More recent studies have shown that this can be reversed by administering a membrane-permeant version of PtdIns-3,4,5-P_3 [163]. In addition, expression of the SHIP phosphatase, which is capable of dephosphorylating PtdIns-3,4,5-P_3, results in inhibition if insulin-stimulated GLUT4 translocation [148]. Similarly, studies using both dominant-negative as well as constitutively active mutants of p85/p110 PI 3-K have proven beyond doubt that PI 3-K is a key regulator of GLUT4 translocation [164]. What has remained more elusive and contradictory is the role played by its effector molecules. Several initial studies provided evidence that Akt/PKB was also required for insulin-stimulated GLUT4 translocation [165], and indeed that Akt/PKB isoforms such as Akt-2 are recruited to GLUT4 [166]. However, more recent studies performed with true dominant-negative Akt/PKB alleles has shown that in fact, Akt/PKB does not appear to be necessary for translocation, but rather the atypical PKCλ and PKCζ isoforms contribute to insulin-stimulated glucose uptake in adipocytes [167]. It remains to be established which of the several currently identified PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 effectors contribute to GLUT4 translocation in a physiologically relevant setting. Of the known small GTPases, Rab4 has also emerged as a candidate for insulin-dependent glucose uptake downstream of PI 3-K, as wortmannin blocks translocation of Rab4-containing microsomes from the membrane to the cytosol [168]. Similarly, the role of other phosphoinositide kinases in mediating glucose uptake has not been critically addressed. With the discovery of FYVE-containing proteins such as EEA1 and their role in vesicle trafficking, it will undoubtedly be interesting to see whether there is a role for these molecules in mediating PI 3-K-dependent GLUT4 translocation.

Conclusions and perspectives

The availability of various tools for phosphoinositide research, such as clones for the various phosphoinositide kinases and phosphatases, clones for their immediate effector molecules, specific inhibitors as well as synthetic phosphoinositides for in vitro biochemistry has resulted in many important discoveries in the field, particularly over the last 10 years. It is likely that the pace of this

research will only accelerate with the advent of better tools to study individual phosphoinositides. Clearly, phosphoinositides regulate a multitude of physiological processes by specifically interacting with individual effector molecules and domains, many of which have now been discovered and characterized. The best studied to date is undoubtedly the PH domain, and phosphoinositide-PH domain interactions are responsible for a variety of biological processes which are initiated at the plasma membrane. However, it noteworthy that many other phosphoinositide-interacting peptide sequences also exist, such as those on actin-binding proteins which mediate interaction with PtdIns-4,5-P_2, and although these are not as well characterized, it suggests that other protein domains which mediate the lipid-protein interaction exist which are yet to be discovered. Although the number of phosphoinositide effector proteins discovered is likely to increase steadily in the next few years, a major challenge in the field will remain attributing physiological functions to these interactions. It is difficult to rationalize how one individual phosphoinositide, PtdIns-4,5-P_2 or PtdIns-3,4,5-P_3, for example, can interact with so many effector molecules to mediate such diverse biological functions. One anticipated resolution to this problem is that different pools of the individual phosphoinositides exist at any one time in the cell, and that the regulation of these pools is tightly regulated both spatially and temporally. How might one both prove the existence of such pools in vivo, and use the resulting information to gain further insight into signaling mechanisms? Clearly, this will require new technologies in lipid signaling. Although isolation of intact distinct phosphoinositide pools has been successful in some systems (e.g. the nuclear phosphoinositide cycle), it is difficult to see how this might be applied to other cellular pools which have no discrete intracellular boundaries. Armed with the biochemical evidence that individual PH domains have high affinity and specificity for one phosphoinositide, this information could be used to visualize the location of an isolated PH domain inside the cell using green fluorescent protein as a marker. Similarly, detection of lipid-protein interactions in intact cells will be useful if only to corroborate the in vitro findings described to date. Another area likely to receive considerable attention is the convergence of different signals to an effector lipid-binding protein. Examples are both Btk and Akt/PKB, which require the synergistic action of PtdIns-3,4,5-P_3 binding and phosphorylation to achieve maximal activation of the protein kinase. The interactions reviewed here have set the stage as well as the precedent for phosphoinositide signaling; the task at hand is to confirm these in a physiological setting, to dissect the complexities of the various signaling pathways and to elucidate the downstream signaling pathways which are initiated by phosphoinositide synthesis at the plasma membrane.

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