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PHOSPHOINOSITIDE KINASES

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ABSTRACT

Phosphatidylinositol, a component of eukaryotic cell membranes, is unique among phospholipids in that its head group can be phosphorylated at multiple free hydroxyls. Several phosphorylated derivatives of phosphatidylinositol, collectively termed phosphoinositides, have been identified in eukaryotic cells from yeast to mammals. Phosphoinositides are involved in the regulation of diverse cellular processes, including proliferation, survival, cytoskeletal organization, vesicle trafficking, glucose transport, and platelet function. The enzymes that phosphorylate phosphatidylinositol and its derivatives are termed phosphoinositide kinases. Recent advances have challenged previous hypotheses about the substrate selectivity of different phosphoinositide kinase families. Here we re-examine the pathways of phosphoinositide synthesis and the enzymes involved.

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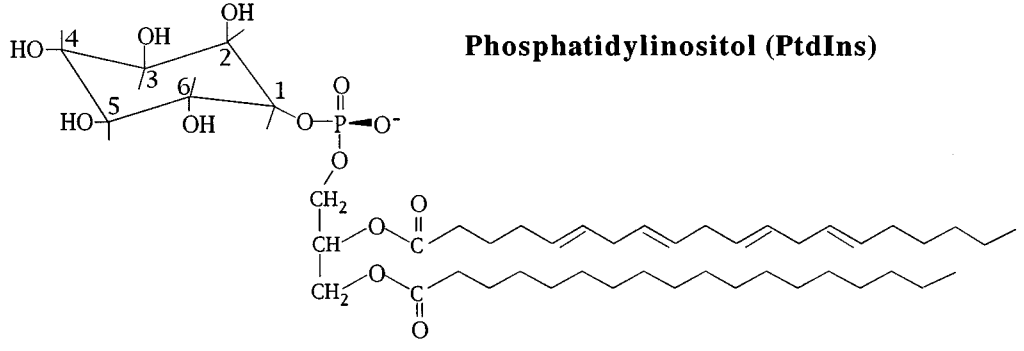
INTRODUCTION

Although phosphatidylinositol (PtdIns) represents only a small percentage of total cellular phospholipids, it plays a crucial role in signal transduction as the precursor of several second-messenger molecules. The inositol head group contains five free hydroxyls with the potential to become phosphorylated (Figure 1*a*). Thus, numerous derivatives of PtdIns[1] could exist in cells, each with a unique function. To date, the following phosphoinositides have been identified in cells: PtdIns-3-phosphate (hereafter termed PtdIns-3-P), PtdIns-4-phosphate (PtdIns-4-P), PtdIns-5-phosphate (PtdIns-5-P), PtdIns-3,4-*bis*phosphate (PtdIns-3,4-P_2), PtdIns-3,5-*bis*phosphate (PtdIns-3,5-P_2), PtdIns-4,5-*bis*-phosphate (PtdIns-4,5-P_2), and PtdIns-3,4,5-*tris*phosphate (PtdIns-3,4,5-P_3) (Figure 1*b*). Three general functions of these lipids could be imagined: (*a*) to serve as phospholipase substrates for the generation of soluble inositol phosphate (and membrane-associated diacylglycerol) second messengers; (*b*) to interact directly with intracellular proteins, affecting their location and/or activity; (*c*) to alter local membrane topology by electrostatic interactions. The first two functions are well established for certain phosphoinositides, and some data provide evidence for the third role. The specific cellular functions of the individual phosphoinositides have recently been reviewed in detail (1–4). Our intention here is to present our current understanding of the pathways of synthesis of each phosphorylated lipid and how different steps are regulated. Although some phosphoinositides can be generated by lipid-specific phosphatases acting upon more highly phosphorylated forms, the lipid kinases are the focus of this review.

Many phosphoinositide kinases were described initially as enzymatic activities capable of transferring a phosphate to a particular position on the inositol ring of PtdIns or one or more of its phosphorylated derivatives. Studies of these purified enzymes led to the categorization of PtdIns or phosphoinositide kinases into three general families: phosphoinositide 3-kinases (PI3Ks), PtdIns 4-kinases (PtdIns4Ks), and PtdIns-P (PIP) kinases (PIP5Ks). Research in this area has been accelerated by the cloning of genes encoding several of these enzyme classes. Members of each phosphoinositide kinase family have been identified in yeast and other lower eukaryotes; each shares substantial protein-sequence homology with its mammalian counterparts. This evolutionary

[1]This review uses nomenclature that distinguishes phosphatidylinositol from phosphoinositides, the phosphorylated derivatives of PtdIns.

a.



b.

Monophosphoinositides:	PtdIns-3-P, PtdIns-4-P, PtdIns-5-P
Bisphosphoinositides:	PtdIns-3,4-P_2, PtdIns-3,5-P_2, PtdIns-4,5-P_2
Trisphosphoinositides:	PtdIns-3,4,5-P_3

Figure 1 (*a*) Chemical structure of phosphatidylinositol (PtdIns). Note the free hydroxyls at positions 2–6 of the inositol head group. The *myo*-D-enantiomer of inositol is shown, in which the 2′-hydroxyl is axial and the other hydroxyls are equatorial. (*b*) List of phosphoinositides known to exist in mammalian cells. PtdIns-4-P and PtdIns-4,5-P_2 represent approximately 60% and 30%, respectively, of the total phosphoinositides in cells. It is important to note that PtdIns itself is not considered a phosphoinositide. Some of the enzymes discussed in this review are not strictly phosphoinositide kinases because they phosphorylate only PtdIns (see text). However, they belong to the phosphoinositide kinase superfamily based on sequence homology.

conservation underscores the importance of these enzymes in the physiology of all eukaryotic cells. Sequence homology generally supports the separate classification of PI3Ks, PtdIns4Ks, and PIP kinases (for reviews of phosphoinositide kinase phylogeny, see References 5–7). However, studies of the recombinant enzymes have revealed that certain phosphoinositide kinases have different or broader activities than realized previously.

Each section that follows begins with a review of the molecular biology of a particular phosphoinositide kinase family and concludes with a discussion of the enzymology and regulation of the family members. For some enzymes we suggest new nomenclature that reflects our current understanding of substrate selectivity. We conclude with an overview of phosphoinositide synthesis pathways, using the proposed nomenclature where appropriate. The reader is referred to several recent reviews that detail the cellular functions of the enzymes and their lipid products (1–4, 8, 9).

PI 3-KINASES

PI3Ks have been studied intensively since the discovery of a PI3K activity associated with two viral oncoproteins: polyoma middle T (mT) antigen and $pp60^{v\text{-}src}$ (10). Subsequent work has confirmed a role for PI3Ks and their products not only in growth regulation but also in various other cellular responses. Interest in these enzymes has further increased following recent findings that PI3K activation prevents cell death (9), that PI3K is a retrovirus-encoded oncogene (11), and that PI3K mutations increase lifespan in *Caenorhabditis elegans* (12). We refer to the different PI3K classes using nomenclature proposed recently by Domin & Waterfield (7).

Class I PI3Ks

MOLECULAR BIOLOGY Class I PI3Ks are heterodimeric proteins, each of which consists of a catalytic subunit of 110–120 kDa and an associated regulatory subunit. Three mammalian PI3Ks sharing 42–58% amino acid sequence identity have been cloned and designated p110α, p110β, and p110δ (13–15). Each of these proteins contains an N-terminal region that interacts with regulatory subunits, a domain that binds to the small G protein ras, a "PIK domain" homologous to a region found in other phosphoinositide kinases, and a C-terminal catalytic domain (Figure 2). p110-related genes have been cloned from a range of eukaryotes including *C. elegans*, *Drosophila melanogaster*, and *Dictylostelium discoideum* (12, 16, 17). Together these gene products are termed class I_A PI3Ks.

The regulatory subunits that associate with class I_A PI3Ks are often called p85 proteins, based on the molecular weight of the first two isoforms to be purified (18) and cloned (19–21): p85α and p85β. p85 proteins do not possess any

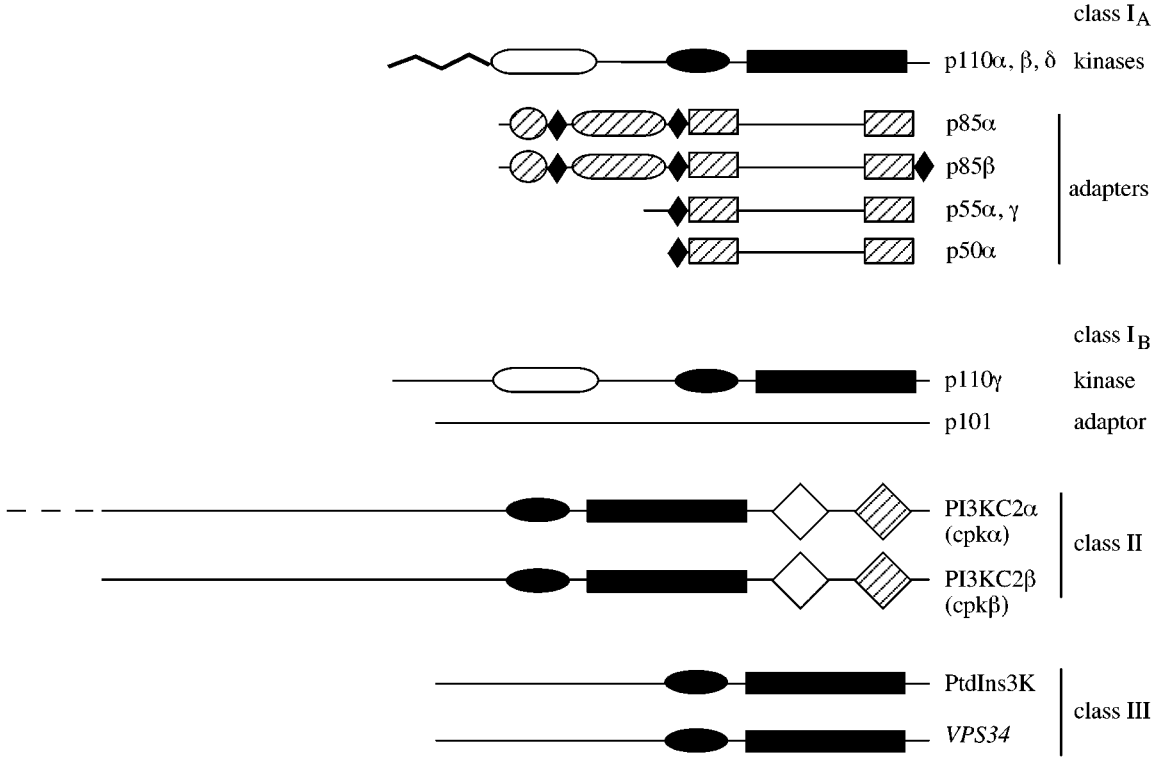


Figure 2 Structural features of phosphoinositide 3-kinase (PI3K) family members. Three classes of mammalian PI3Ks have been defined; class I is further subdivided based on the associated adapter (regulatory) subunit. The only yeast PI3K, Vps34, is homologous to the human phosphatidylinositol (PtdIns)-specific class III enzyme (denoted PtdIns 3-kinase). The protein domains are as follows: catalytic domain (*solid rectangles*), phosphoinositide kinase (PIK) domain (*solid ovals*), ras-binding domain (*open ovals*), rhoGAP-homology domain (*hatched oval*), SH2 domain (*hatched rectangles*), SH3 domain (*hatched circle*), proline-rich motif (*solid diamonds*), C2 domain (*hatched diamonds*), PX domain (*open diamonds*), p85-interaction region in class IA enzymes (*sawtoothed line*).

known enzymatic activity but are composed of several domains with homology to those found in other signaling proteins. These domains are termed modular because they can be separated functionally and spatially from the rest of the protein in which they reside. p85α and p85β contain an N-terminal src-homology 3 (SH3) domain, two or three proline-rich segments, a region of homology to GTPase-activating proteins for the rho family of small G proteins (rho-GAPs), and two src-homology 2 (SH2) domains (Figure 2). The function of each of these modules is discussed below. Between the two SH2 domains is a region that is necessary and sufficient for interaction with the N terminus of p110 catalytic subunits. Termed the inter-SH2 domain, this region contains sequences that are predicted to form α-helices that fold into a coiled-coil structure.

The p85α gene has several splice variants, two of which encode the smaller proteins p50α and p55α (22–25). These proteins have unique N termini of 6 and 35 amino acids, respectively, and share the C terminus of p85α, including the second proline-rich motif, the SH2 domains, and the inter-SH2 domain (Figure 2). A third gene, p55γ, encodes a protein with similar overall structure to p55α (26). There is no evidence to date that different regulatory subunit isoforms pair preferentially with different p110 isoforms (15). However, some data suggest that different p85 subunits may associate with different subsets of intracellular proteins (27–29). *Drosophila* contain a p85-related protein of 60 kDa with conserved SH2 domains and an inter-SH2 domain with probable coiled-coil structure (30).

A protein with 36% identity to p110α was cloned and designated p110γ (31–33). p110γ contains a PIK domain, a kinase domain, and a ras-binding domain but diverges from class I_A PI3Ks at its N terminus (Figure 2) and does not interact with p85 proteins. It has therefore been designated a class I_B PI3K. p101, a putative regulatory subunit for p110γ, possesses no recognizable homology to other proteins (33). The regions of interaction between p101 and p110γ have not been mapped.

ACTIVITY AND REGULATION In vitro, all the class I PI3Ks are able to phosphorylate PtdIns, PtdIns-4-P, or PtdIns-4,5-P_2 on the free 3-position (Table 1). Class I_A PI3Ks also phosphorylate PtdIns-5-P in vitro (34). However, agonists that stimulate these enzymes in vivo cause increases mainly in the cellular levels of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3. In addition, kinetic studies in ^{32}P-labeled cells suggest that PtdIns-3,4-P_2 may be produced in part by the action of a 5-phosphatase on PtdIns-3,4,5-P_3 (35). Thus, the class I PI3Ks may be selective for PtdIns-4,5-P_2 in vivo. Of note, the inter-SH2 domain of p85α binds to PtdIns-4-P and PtdIns-4,5-P_2 in vitro (36); this property may provide a mechanism for presenting these substrates to the catalytic subunit or to concentrate the enzyme at membranes rich in these lipids.

Table 1 Properties of mammalian phosphoinositide kinases

Enzyme	Catalytic subunits: Isoforms	Catalytic subunits: MW (kDa)[a]	Regulatory subunits	In vitro substrates	Inhibitors[b]
Class I_A PI3K	p110α p110β p110δ	123 (110) 123 (119) 119 (115)	p85α, p55α, p50α, p85β, p55γ[c]	PtdIns-4,5-P_2, PtdIns-4-P, PtdIns,[d] PtdIns-5-P	Detergent, wortmannin (1–10), Ly294002 (1000)
Class I_B PI3K	p110γ	120 (110)	p101	PtdIns-4,5-P_2, PtdIns-4-P, PtdIns[d]	Detergent, wortmannin (1–10), Ly294002 (1000)
Class II PI3K	P13KC2α P13KC2β	170, 210 180		PtdIns, PtdIns-4-P, (PtdIns-4,5-P_2)[e]	Wortmannin (50–450), Ly294002 (19000)
Class III PI3K (PtdIns3K)		101	p150	PtdIns	Wortmannin (2–10)
PtdIns4Kα		100, 230[f]		PtdIns	Adenosine 4C5G antibody Wortmannin?[f]
PtdIns4Kβ		90 (110)		PtdIns	Wortmannin (150)
PIP4K	α and β	47 (53)		PtdIns-5-P, PtdIns-3-P	PtdIns-4,5-P_2 Heparin
PIP5K	α and β	61 (68)		PtdIns-4-P, PtdIns-3,4-P_2, PtdIns-3-P[g]	PtdIns-4,5-P_2

[a]The deduced molecular weight (MW) of each phosphoinositide kinase is shown. Numbers in parentheses represent cases where the apparent molecular weight determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is significantly different.

[b]Values in parentheses indicate the 50% inhibitory concentration (IC_{50}) or range of IC_{50} values (in nanomolar concentration) of the inhibitor.

[c]Most of the possible combinations of class I_A catalytic and regulatory subunits have been demonstrated to occur in vivo (15).

[d]Each class I enzyme can utilize all three lipid substrates in vitro, although phosphatidylinositol-4,5-*bis*phosphate (PtdIns-4,5-P_2) appears to be the preferred substrate in vivo.

[e]PtdIns is the preferred substrate of class II enzymes in vitro. PtdIns-4,5-P_2 is phosphorylated only when phosphatidylserine is used as a carrier.

[f]The two PtdIns4Kα proteins appear to be products of alternative splicing. The higher-molecular-weight form has been reported to be inhibited by ~200 nM wortmannin (116).

[g]PtdIns-3-P can be converted to PtdIns-3,4,5-P_3 in an apparent concerted reaction (133).

Class I PI3Ks possess intrinsic protein kinase activity that is inseparable from their lipid kinase activity (15, 37–39). In fact, all phosphoinositide kinases contain several key residues conserved in the catalytic domains of classical protein kinases. The major substrates of the protein kinase activity of a class I PI3K are serine residues within the catalytic subunit itself and/or its associated regulatory subunit. p110α phosphorylates p85α at serine 608; interestingly, this phosphorylation results in down-regulation of the lipid kinase activity of p110α (37, 38). p110δ prefers autophosphorylation to intersubunit phosphorylation, but this autophosphorylation similarly down-regulates enzyme activity (15). p110γ also autophosphorylates, but without demonstrably affecting enzyme activity (39).

p85/p110 complexes can phosphorylate insulin receptor substrate-1 (IRS-1) in vitro and possibly in vivo (40, 41); however, other protein substrates for class I PI3Ks in vivo have not been established.

The fungal metabolite wortmannin is a potent inhibitor of the lipid (and protein) kinase activities of class I PI3Ks. The 50% inhibitory concentration (IC_{50}) values for inhibition of the isolated enzymes are all in the range of 1–10 nM (Table 1). Similar concentrations are required to inhibit p85/p110 PI3K in vivo, as judged by effects on the activity of the enzyme immunoprecipitated from cells treated with the drug. Wortmannin irreversibly inhibits p110α by reacting covalently with lysine-802 (42), a residue required for catalytic activity that is conserved in all phosphoinositide kinases (and in protein kinases). A second pharmacological PI3K inhibitor, Ly294002, is a reversible inhibitor of class I enzymes with IC_{50} values of approximately 1 μM (43). Wortmannin and Ly294002 have been used extensively to study the physiological role of class I PI3Ks in various cellular responses. However, some of these studies should be interpreted with caution owing to the emerging evidence that at somewhat higher concentrations these compounds inhibit other signaling enzymes, including PtdIns4Kβ (44) and the related protein kinases TOR (45) and DNA-PK (46).

Class I_A PI3Ks are regulated by interaction with the small G protein ras. Many extracellular stimuli activate ras by increasing the ratio of bound GTP to GDP. In turn, ras-GTP interacts with a number of downstream "effectors." p110α has been established as a ras effector: Its activity is increased in vitro and in vivo by ras-GTP, dominant negative forms of ras can interfere with 3′-phosphorylated phosphoinositide production, and ras effector domain mutants that fail to interact with PI3K are defective in certain ras-dependent cellular responses (47–50).

The activities and subcellular locations of Class I_A PI3K catalytic subunits are also regulated by p85 proteins. p85 and its relatives are sometimes referred to as adapter subunits because they possess several modular domains with the capacity to interact with other signaling proteins. The SH2 domains of p85 have been studied in detail. Like SH2 domains in other signaling proteins, they bind selectively to phosphotyrosyl (pTyr) residues within specific sequence contexts. In all known p85 proteins, both the N-terminal SH2 (N-SH2) and C-terminal SH2 (C-SH2) domains bind preferentially to polypeptides containing a p-Tyr-X1-X2-Met motif (51). A second methionine or valine at the X1 position increases binding affinity, particularly for the N-SH2 domain (51). A crystal structure of the N-SH2 domain bound to a phosphopeptide explains the binding selectivity (52). A solution structure of the C-SH2 domain has also been determined by nuclear magnetic resonance (53). Synthetic peptides containing tandem pTyr-Met-X-Met (pYMXM) motifs separated by a spacer region bind

with high affinity to p85 proteins and, importantly, increase the catalytic activity of the associated p110 subunits two- to threefold in vitro (54, 55).

Many stimuli trigger phosphorylation of YMXM motifs, which recruits p85-p110 complexes and thereby enhances PI3K activity (discussed in detail in 8). Most of the agonists that activate p110 via p85/pYMXM interactions also activate ras, itself a p110 activator, as discussed above. For example, polyoma mT has a YMXM sequence to interact with PI3K adapter proteins and other tyrosine residues that mediate interactions leading to activation of ras. Variant polyoma mT proteins that fail to activate ras are unable to increase production of cellular 3′-phosphorylated phosphoinositides, even if the YMXM motif is intact (56, 57). Many YMXM-containing proteins are membrane associated, as is ras. Therefore, recruitment of p85/p110 complexes not only increases catalytic activity but also brings the PI3K from the cytoplasm to the membrane, where its substrates and a potential activator (ras) reside.

Interestingly, the SH2 domains of p85 proteins also bind to phosphoinositides in vitro and exhibit marked selectivity for PtdIns-3,4,5-P_3 (58). Lipid binding to the SH2 domains competes with the binding of pTyr-containing peptide, which suggests that the production of PtdIns-3,4,5-P_3 by activated PI3K causes dissociation of the p85/phosphopeptide complex from its pYMXM docking sites. This model is supported by the finding that wortmannin treatment stabilizes p85/pTyr interactions in cells (58). It is also possible that lipid binding regulates enzyme activity allosterically or by influencing its membrane attachment.

SH3 domains are known to interact with proline-rich sequences with the consensus motif φ–P-p-φ-P, where P is an invariant proline, p is a weakly conserved proline, and φ is an aliphatic amino acid (59). This motif forms a left-handed type II polyproline helix that fits into a hydrophobic platform in the SH3 domain (60). Solution structures have been determined for the p85α SH3 domain alone or bound to a high-affinity peptide ligand (61, 62). A GST fusion protein of p85α SH3 selected several candidate partner proteins from bovine brain extracts, including the GTPase dynamin (61). In addition, the p85α-SH3 domain may interact with proline-rich motifs within the p85 protein itself (63). Intramolecular association of these two modules may prevent either of them from finding other, higher-affinity partners in unstimulated cells.

The proline-rich motifs of p85α mediate binding to SH3 domains of src family kinases including src itself, lck, lyn, and fyn (63–69). The SH3 domain of the cytoplasmic tyrosine kinase abl also associates with p85α (63). For binding to lyn and fyn, the N-terminal proline-rich motif of p85α is more effective than the C-terminal motif (69). The proline-rich regions of p85β diverge considerably in sequence from the analogous regions of p85α. In addition, p85β possesses a third PPXP motif between the C-SH2 domain and the C terminus. Thus, p85β may select a different set of SH3-containing proteins in vivo. The p50α, p55α,

and p55γ regulatory subunits contain only a single polyproline motif and lack the N-terminal motif that is selective for binding src.

The binding of protein kinases of the src family via their SH3 domains to the proline-rich motifs of p85 does not depend on any posttranslational modification. This fact suggests that the p85-kinase interaction is regulated by a different mechanism. The crystal structures of src and hck in their inactive states shows that the endogenous SH3 domain makes intramolecular contact with a cryptic polyproline-like helix formed by the spacer sequence between the SH2 and kinase domains (70, 71). Thus, intermolecular association of src family kinases and p85 proteins may first require release of intramolecular contacts within each protein. Association of p85α with the phosphoprotein cbl is also consistent with this type of model. Binding of pTyr residues on cbl to the SH2 domains of p85α appears to expose the SH3 domain to allow high-affinity interactions with proline-rich regions of cbl (72).

The rho-GAP homology region—originally termed the breakpoint cluster region (bcr)-homology domain—of p85α lacks GTPase-promoting activity in vitro toward the small G proteins rho, rac, and cdc42. The crystal structure of the p85α rho-GAP domain is similar to bona fide rho-GAPs but lacks five conserved residues likely to be important for catalysis (73–75). Nevertheless, the p85α rho-GAP domain binds to rac and cdc42 in vitro in a GTP-dependent manner (76, 77). It is possible that rac and/or cdc42 regulates p85/p110 complexes or helps locate these complexes at specific regions of the cell (or vice versa). Because the rho-GAP homology region of p85α lies between its SH3 domain and its second proline-rich motif in the primary structure, binding of a small G protein may either disrupt or enhance the intramolecular interactions discussed above. The rho-GAP region of p85β is only 42% identical to the corresponding domain of p85α, and it similarly lacks residues thought to be important for GTPase-promoting activity (20, 73).

p110γ differs from other class I enzymes because of its ability to be directly activated by $\beta\gamma$ subunits of heterotrimeric G proteins (31, 32, 78, 79). Many G protein–coupled serpentine receptors increase the levels of various 3′-phosphorylated PtdIns species upon ligand binding. Recent evidence has shown that these increases are mediated by p110γ (78). One group found that G$\beta\gamma$ subunits could activate p110γ directly (32), whereas another reported that the associated p101 subunit binds G$\beta\gamma$ and is required for significant activation (33). Interestingly, a p85/p110-type PI3K activity was also shown to be activated by G$\beta\gamma$ subunits, but only in the presence of pTyr peptides that bind to the SH2 domains of the adapter subunit (79, 80). p110γ/p101 activity is not affected by phosphotyrosyl peptides. p110γ also contains a ras-binding domain and associates with ras in vitro (80a). However, a role for ras in p110γ activation has not been demonstrated.

Class II PI3Ks

MOLECULAR BIOLOGY Class II PI3Ks are large (170–210 kDa) proteins that contain a PIK domain and a catalytic domain 45–50% similar to class I PI3Ks (Figure 2). Class II PI3K genes have been cloned from humans and mice as well as from *D. melanogaster*, *D. discoideum*, and *C. elegans* (17, 81–84). Each of these proteins contains a C-terminal region with homology to C2 domains; indeed, the class II PI3Ks have been termed PI3KC2 (used herein) or cpk (for C2-containing phosphoinositide kinase). Other proteins with C2 domains include certain protein kinase C (PKC) isoforms and the synaptic vesicle membrane protein synaptotagmin. C2 domains have been implicated in the Ca^{2+}-dependent binding of proteins to lipid vesicles. However, certain Asp residues important for Ca^{2+} binding are absent in the C2 domains of class II PI3Ks. The *Drosophila* PI3KC2 was found to bind acidic phospholipids in a Ca^{2+}-independent manner (81). Mammalian PI3KC2α and the novel human PI3KC2β have an additional sequence motif termed the PX domain (see Figure 2) (S Volinia, personal communication). Homologous domains have been noted in several signaling proteins, including the NADPH oxidase-associated proteins phox-40 and phox-47. The function of PX domains is unknown.

ACTIVITY AND REGULATION In vitro, class II PI3Ks preferentially phosphorylate PtdIns and PtdIns-4-P (Table 1), although human PI3KC2α phosphorylates PtdIns-4,5-P_2 in the presence of phosphatidylserine (84). The enzymes from *Drosophila*, mouse, and human differ significantly in sensitivity to inhibition by wortmannin (IC_{50} values of 5, 50, and 450 nM, respectively) (81, 83, 84) (Table 1). It has not yet been determined which lipids are produced by class II PI3Ks in vivo and how their activities are regulated. If these enzymes make PtdIns-3,4-P_2 in vivo, they are not likely to be active in resting cells where this lipid is undetectable. It is possible that class II PI3Ks contribute to the buildup of PtdIns-3,4-P_2 observed in stimulated cells. There is evidence for rapid and transient tyrosine phosphorylation of PI3KC2α and -β following mitogen stimulation (82; S Volinia, personal communication), but the effect on enzyme activity is unknown.

Class III PI3Ks

MOLECULAR BIOLOGY The prototype class III PI3K was first identified in yeast in a screen for mutants conditionally defective in vacuolar protein sorting (85). The corresponding gene, *VPS34*, was cloned and found to be essential for accurate transport of newly synthesized proteins from the Golgi to the vacuole, an organelle similar to the lysosome of higher eukaryotes. The lipid kinase activity of this yeast protein was appreciated only after cloning of the p110 subunit of mammalian class I PI3Ks revealed that the proteins shared extensive

sequence homology (86) (Figure 2). Bona fide *VPS34* homologues have now been cloned from humans, *Dictyostelium*, and *Drosophila* (17, 87, 88). Each of the proteins also possesses a PIK domain (Figure 2).

ACTIVITY AND REGULATION Class III PI3Ks phosphorylate only PtdIns (Table 1); therefore they should be called PtdIns 3-kinases to differentiate them from the phosphoinositide 3-kinases with broader substrate specificity. Although the yeast Vps34 is relatively insensitive to wortmannin ($IC_{50} \sim 2.5\ \mu M$), human and *Drosophila* Vps34 homologues are inhibited with IC_{50} values of 2–10 nM (Table 1). In addition, wortmannin treatment lowers the level of PtdIns-3-P in platelets (89, 90).

The yeast protein Vps34 associates with another protein, Vps15, that is also required for vesicle sorting (91). Vps15 is a serine/threonine kinase that recruits Vps34 to membranes and enhances its lipid kinase activity. A human DNA encoding of protein with 30% sequence identity to the *VPS15* gene product was cloned. This protein was found to act as an adapter for the human class III PI3K (92). The human heterodimeric class III PI3K was found to associate with phosphatidylinositol transfer protein, which stimulated lipid kinase activity (92). That PtdIns-3-P levels are comparable in both resting and stimulated cells (90) suggests that class III PI3Ks induce local increases in PtdIns-3-P that may be required for agonist-independent membrane trafficking processes.

PtdIns 4-KINASES

The existence of PtdIns 4-kinases (PtdIns4Ks) has been appreciated for some 30 years, yet our understanding of these enzymes has been limited. The genes encoding the enzymes have only recently been cloned. PtdIns4Ks convert PtdIns to PtdIns-4-P by phosphorylating the inositol ring at the D4 position. The PtdIns-4-P thus generated can be further phosphorylated by both PI3Ks and PtdIns-4-P 5-kinases (PIP5Ks) to yield PtdIns-3,4-P_2 or PtdIns-4,5-P_2, respectively. In this way, the PtdIns4Ks play a central role in signaling by feeding into multiple pathways. These enzymes are ubiquitously expressed and are most abundant in cellular membranes including the Golgi, lysosomes, endoplasmic reticulum (ER), plasma membrane, and a variety of vesicles that include Glut 4–containing vesicles, secretory vesicles, and coated pits (93). Unlike the functionally related PI3Ks and the PIP5Ks, these enzymes appear to use only PtdIns as a substrate and cannot phosphorylate either the singly or doubly phosphorylated lipids generated by the other enzymes.

The PtdIns4Ks were classically subdivided into two types (II and III) based on biochemical differences of the partially purified enzymes (94, 95). Recently, several PtdIns4Ks have been cloned from yeast, mammals, and other species.

Characterization of the enzymes that have been cloned from both yeast and mammalian cells has revealed additional subtypes of PtdIns 4-kinases. In the next section we describe the properties of the purified enzymes and then describe the structure and enzymology of the cloned PtdIns 4-kinases.

Purification of the 4-Kinases

Type II PtdIns4Ks were defined biochemically as single subunit, membrane-bound enzymes whose lipid kinase activity is stimulated by nonionic detergent and inhibited by adenosine (95). They were purified from a variety of mammalian sources, including fibroblasts, bovine brain, A431 cells, and erythrocytes (94–97). The type II PtdIns4K was characterized as a 55-kDa protein that could be renatured from a sodium dodecyl sulfate (SDS) gel (96, 98) and could be inhibited by the monoclonal antibody 4C5G (99). Although this activity is the most potent PtdIns4K in most cell types, the gene has not yet been cloned.

The p55 enzyme has been implicated in many signaling pathways through its association with a variety of molecules and subcellular compartments, including epidermal growth factor (EGF) receptor (100), chromaffin granules (101), CD4-p56^{lck} (102), integrins (103), and PKCs. Unfortunately, a more detailed analysis of the role of this enzyme in these pathways awaits the cloning of the gene encoding the p55 type II enzyme.

Type III PtdIns4Ks were defined as membrane-bound enzymes whose lipid kinase activity is unaffected by high concentrations of adenosine and is maximally active in nonionic detergent (95). The enzyme, purified from bovine and rat brain, has an apparent molecular weight of 220 kDa by sucrose gradients and gel filtration (95, 104), and it is resistant to the monoclonal antibody 4C5G (99).

The prototype PtdIns4Ks were first cloned from yeast and designated *PIK1* (114) and *STT4* (106). Subsequently, cDNAs for two mammalian PtdIns4Ks were cloned; their properties are described below. The encoded enzymes do not strictly follow the typing described above and are therefore called PtdIns4Kα and PtdIns4Kβ, based on the order in which they were isolated from mammalian sources.

PtdIns4Kα/STT4

MOLECULAR BIOLOGY PtdIns4Kα is homologous to a yeast PtdIns4K, the *STT4* gene product that was identified as a staurosporine- and temperature-sensitive mutant in yeast (105). Stt4 is a ~210-kDa protein with a conserved C-terminal catalytic domain that is ~35% identical to PtdIns4Kβ and ~27% identical to the PI3Ks (106). Null mutants (*stt4*Δ) are lethal unless supplied with an exogenous osmotic support (1M sorbitol). Under these conditions, the cells display decreased doubling times and a fivefold decrease in the level of PtdIns-4-P (106). Additionally, overexpression of a protein kinase C gene

(*PKC1*) can complement the staurosporine sensitivity of an *stt4-1* mutant, which suggests that *PKC1* is downstream of *STT4* in yeast (107). However, PKC enzymes bind staurosporine, so this rescue could be indirect, because *PKC1* overexpression does not rescue any of the other phenotypes of *sst4Δ* mutants.

PtdIns4Kα was cloned from a human placenta cDNA library. It encodes an 854-amino-acid protein (p97) that is 50% identical to Stt4 in the catalytic domain and is also more similar to Stt4 than to Pik1 in the noncatalytic region (108). Homologues have now been cloned from rat and bovine brain (109, 110). Interestingly, the rat brain protein shares 98% identity with human p97 PtdIns4Kα over the region of overlap but encodes a protein of 2041 amino acids (p220) with a distinct N-terminal half. We refer to these genes as p97 PtdIns4Kα and p220 PtdIns4Kα. In addition to the C-terminal catalytic domain, p97 and p220 PtdIns4Kα contain an amino-proximal PIK domain (Figure 3) as well as regions with possible homology to ankyrin repeats. The unique N-terminal portion of p220 PtdIns4Kα contains a proline-rich segment and an SH3 domain (111) (Figure 3). The p97 PtdIns4Kα enzyme is likely to be a spliced variant of the larger protein because antibodies against p97 cross-react with a larger protein in human cells (112). By RNA hybridization and immunoblotting, most cells appear to express predominantly p220 PtdIns4Kα with the noted exception of platelets, which express significant amounts of p97 PtdIns4Kα (K Wong, personal communication).

ACTIVITY AND REGULATION In vitro, the PtdIns4Kα/Stt4 isoforms phosphorylate only PtdIns. Although PtdIns4Kα is relatively resistant to wortmannin (K Wong, personal communication), recent data suggest that the p220 PtdIns4Kα protein can be completely inhibited at a high concentration of

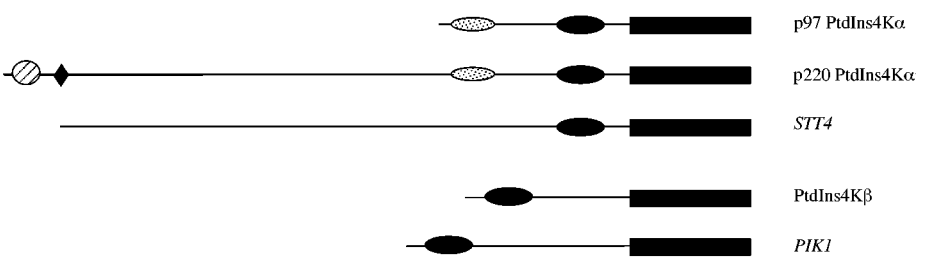


Figure 3 Structural features of phosphatidylinositol 4-kinases (PtdIns4K) family members. Two types of PtdIns4K genes have been cloned from yeast and mammals. Mammalian PtdIns4Kα is most closely related to yeast Stt4, whereas mammalian PtdIns4Kβ is more homologous to yeast Pik1. The protein domains are as follows: catalytic domain (*solid rectangles*), phosphoinositide kinase (PIK) domain (*solid ovals*), SH3 domain (*hatched circle*), proline-rich motif (*solid diamond*), ankyrin-like repeats (*stippled ovals*).

wortmannin (10 μM) (109). By contrast, the yeast Stt4 is >90% inhibited by 10 nM wortmannin in vitro (113). In most mammalian cells, the levels of PtdIns-4-P do not appreciably change in response to growth factors or osmotic stress, which suggests that if PtdIns4Ks are regulated by extracellular signals, the regulation is likely to be at specific regions of the cell. Indeed, recent studies have localized the mammalian PtdIns4Ks—PtdIns4Kα and PtdIns4Kβ—to distinct intracellular membranes (ER and Golgi, respectively), where they may regulate the local levels of PtdIns-4-P (111, 112).

PtdIns4Kβ/PIK1

MOLECULAR BIOLOGY Protein purification (117) and peptide sequencing led to the cloning of the first PtdIns4K, *PIK1*, from yeast (114). Pik1 is a 125-kDa enzyme that is essential for *Saccharomyces cerevisiae* survival. It has a catalytic domain that is 30% identical to the catalytic domains of the PI3K family members, and it has a PIK domain (Figure 3). Null mutants (*pik1*Δ) are not viable (114), and temperature-sensitive (ts) alleles show defects in cytokinesis, with most cells appearing as pairs with fully separated nuclei (115). Additionally, it has been reported that *pik1* ts mutants show an approximately threefold decrease in PtdIns4K activity in the nuclear fraction (115).

Recently, cDNAs encoding homologues of *PIK1* have been cloned from rat (109), human (44), and bovine (116) tissues. These enzymes, variously named 92kPtdIns4K, PtdIns4Kβ (used herein), and PtdIns4KIIIβ, are highly related and almost certainly are the same gene product. A *D. discoideum* gene highly related to this family has also been cloned (17). The PtdIns4Kβ/*PIK1* family of enzymes is distinguished from the PtdIns4Kα/*STT4* family and the PI3K family in that the PIK domain is at the N terminus rather than adjacent to the catalytic domain in sequence (Figure 3).

Both Pik1 and PtdIns4Kβ are mostly soluble, cytosolic enzymes (112, 117), although some reports suggest that Pik1 is nuclear (115), and a significant fraction of mammalian PtdIns4Kβ is associated with the Golgi (112).

ACTIVITY AND REGULATION PtdIns4Kβ/Pik1 enzymes phosphorylate only PtdIns to generate PtdIns-4-P (Table 1). The mammalian enzyme is inhibited by wortmannin with an IC_{50} of ~50–100 nM (44, 118, 119), whereas the yeast enzyme is resistant to as much as 5 μM wortmannin (R Meyers, unpublished data; J Thorner, personal communication). Interestingly, wortmannin treatment of adrenal glomerulosa cells leads to a decrease in the hormone-stimulated production of PtdIns-4-P and PtdIns-4,5-P_2 (118). Furthermore, 10 μM but not 300 nM wortmannin inhibits the sustained increase in both IP_3 production and cytoplasmic Ca^{2+} in agonist-stimulated adrenal glomerulosa and Jurkat cells. These results suggest a role for a wortmannin-sensitive PtdIns4K (but not PI3K)

in regulation of agonist-sensitive pools of PtdIns-4,5-P_2 in these cells. Because the p55 type II PtdIns4K is not affected by high concentrations of wortmannin, these results suggest that PtdIns4Kβ (or PtdIns4Kα—see above) may account for a significant fraction of the lipid pool utilized in agonist-stimulated phosphoinositide turnover in these cells (118, 119). In contrast, B cells treated with 25 nM wortmannin (which inhibits PI3K but not PtdIns4K) showed inhibition of the initial as well as the sustained increases in both IP_3 production and cytoplasmic Ca^{2+}, thus implicating PI3Ks in regulation of IP_3 production in this cell type (120).

OTHER PROTEINS WITH HOMOLOGY TO PtdIns KINASES AND PHOSPHOINOSITIDE KINASES

The conserved catalytic domain present in PI3Ks and PtdIns4Ks is shared by a more distantly related family of proteins. These molecules include a group of gene products that control cell cycle progression in response to DNA damage (*RAD3*, *MEC1*, *TEL 1*, ATM, and ATR) as well as the DNA-activated protein kinase (DNA-PK) and the rapamycin/FKBP-binding proteins *TOR*/FRAP/RAFT1 (121–123). These enzymes are more closely related to each other than to the PI3K and PtdIns4K genes and lack a definitive PIK element. Several of these proteins, including DNA-PK, FRAP, and ATM, have demonstrable protein kinase activity (46, 124, 125). There are reports that the yeast protein Tor2 and its mammalian homologue RAFT1 display PtdIns4K activity on purification from cells (126, 127). The nature of this PtdIns4K is unclear because it shares properties with both type II and type III PtdIns4K. Although immunoprecipitates of a ts mutant of yeast Tor2 showed greatly reduced levels of PtdIns4K activity (127), PtdIns4K activity in Tor2/RAFT1/FRAP is more likely to be the result of the presence of an associated lipid kinase. Consistent with this idea, rapamycin does not affect the lipid kinase activity of Tor2/RAFT1/FRAP or alter the levels of lipids in [^{3}H]-inositol-labeled cells (126–128). Additionally, equal amounts of PtdIns4K activity are detected from immunoprecipitates of wild-type and kinase-dead recombinant FRAP/RAFT1 isolated from SF9 insect cells or mammalian cells (124). More work is needed to address the catalytic activities of these important enzymes.

PtdIns-P KINASES

Two distinct families of enzymes have been purified based on their ability to phosphorylate PIP to produce PtdIns-4,5-P_2. Genes encoding these two families of PIP kinases have now been cloned and found to contain significant sequence

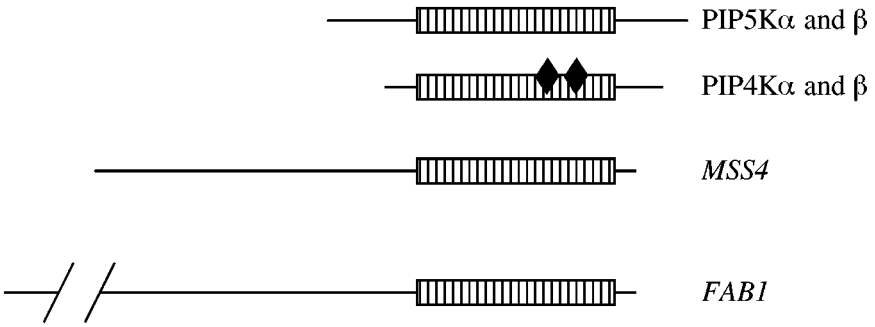


Figure 4 Structural features of PtdIns-4-P 5-kinases (PIP5Ks) and phosphatidylinositol-phosphate 4-kinases (PIP4Ks). Two isoforms of both PIP5K (formerly termed type I PIP5K) and PIP4K (formerly type II PIP5K) have been cloned. Despite their different enzymatic activities, they share significant homology in their putative catalytic domains (*striped rectangles*). The PIP4Ks contain polyproline motifs (*solid diamonds*). Two large proteins in yeast, Mss4 and Fab1, possess domains related to the mammalian enzymes, but their enzymic activities have not been determined. Both PIP4Ks and PIP5Ks are more closely related to Mss4 than to Fab1 (131).

similarity (129–132). Although these enzymes were previously termed type I and type II PtdIns-4-P 5-kinases (PIP5Ks), recent studies have revealed that the two families of enzymes selectively phosphorylate different positions on the inositol ring (34, 133). To reflect the distinct sites of phosphorylation, the two families have been designated PIP5Ks and PIP4Ks, as discussed below.

PIP5Ks

MOLECULAR BIOLOGY Two human PIP5Ks (PIP5Kα and -β) have been cloned. Two groups cloned these genes but used the α and β suffix in a reciprocal manner (130, 131) (Figure 4). We use the designation of Ishihara et al (130). PIP5Kα and -β are 61-kDa proteins migrating at 68 kDa upon SDS-polyacrylamide gel electrophoresis (PAGE) and sharing 64% identity overall and 83% identity in their catalytic domains. Interestingly, type I PIP5Ks exhibit little homology to PI3Ks and PtdIns4Ks. Splice variants of the PIP5Kβ gene have been identified by cDNA cloning and RNA hybridization (131); some of these variants may represent 90-kDa and 110-kDa proteins detected by an anti-PIP5K antiserum (6).

The kinase domains of PIP5Ks are related to two proteins in *S. cerevisiae:* Mss4 and Fab1 (Figure 4). The enzymatic activities of these yeast proteins have not been determined. *MSS4* is an essential gene in yeast, whereas mutations in *FAB1* cause defects in morphology and vacuolar function (107, 134). Genetic evidence places *MSS4* downstream of the yeast PtdIns 4-kinase *STT4*, consistent with Mss4 acting as a PtdIns-4-P kinase (107). Genetic relationships between

the *FAB1* gene and either *STT4* or *PIK1*, the other yeast PtdIns4K, have not been established. The human PIP5Ks are more closely related to Mss4 (40–44% identity) than to Fab1 (27–29% identity) (131). A *C. elegans* gene homologous to *FAB1* (40% identity) has also been identified (131), which suggests that a more highly related mammalian homologue may also exist.

ACTIVITY AND REGULATION The best-studied reaction catalyzed by PIP5Ks is the conversion of PtdIns-4-P to PtdIns-4,5-P_2. Using enzyme purified from erythrocytes, the K_m for PtdIns-4-P in micelles, liposomes, or native membranes ranges from 1 to 10 μM (6). This reaction is stimulated by heparin and spermine and inhibited by the product, PtdIns-4,5-P_2. Importantly, PIP5Ks are stimulated as much as 50-fold by phosphatidic acid (135, 136). This lipid can be generated from phosphatidylcholine by phospholipase D and from diacylglycerol (DAG) by DAG-kinases.

PIP5Ks have been shown to phosphorylate other phosphoinositides in vitro. The α and β isoforms convert PtdIns-3,4-P_2 to PtdIns-3,4,5-P_3 with K_{cat}/K_m ratios that are 3-fold and 100-fold lower, respectively, than those observed using PtdIns-4-P as the substrate (133). Type I PIP5Ks also utilize PtdIns-3-P as a substrate in vitro (133; K Tolias, personal communication). Zhang et al (133) identified the PtdIns-*bis*phosphate product as PtdIns-3,4-P_2; in contrast, work in our laboratory has shown that the *bis*phosphorylated product includes PtdIns-3,5-P_2 (K Tolias, personal communication), a lipid that was recently demonstrated to exist in vivo (137, 137a). Pulse-labeling experiments indicated that most of this lipid is generated by phosphorylation of PtdIns-3-P at the 5′ position (137), a reaction that could be catalyzed by a PIP5K.

Remarkably, PtdIns-3,4,5-P_3 is also produced when recombinant PIP5Ks are mixed with PtdIns-3-P (133; K Tolias, personal communication). The amount of PtdIns-3,4,5-P_3 produced by PIP5Kα is significant, approaching 50% as much as the PtdIns *bis*phosphate produced in a 10-min reaction (133). The mechanism of this reaction and its relevance for lipid production in vivo are unclear. It is not known whether the enzyme generates PtdIns-3,4,5-P_3 by a true concerted reaction in which the substrate remains bound to the enzyme during both phosphorylation steps or whether a PtdIns-*bis*phosphate is released and bound again. One possibility is that the enzyme makes both PtdIns-3,5-P_2 and PtdIns-3,4-P_2 from PtdIns-3-P; these initial products could then be phosphorylated again to produce PtdIns-3,4,5-P_3, although PtdIns-3,5-P_2 may be a very poor substrate for further phosphorylation. In any case, it now appears that the enzymes known as type I PIP5Ks do more than just phosphorylate PtdIns-4-P.

Type I PIP5K activity in vivo may be influenced by small G proteins. The non-hydrolyzable GTP analog GTPγS increases PIP5K activity in several cell types (6). PIP5K activity associates constitutively with rac1 (77) and rhoA (138). The

interaction with rac1 requires a carboxyterminal basic region of this small G protein (139). Addition of rac-GTP to permeabilized platelets or to neutrophil extracts causes increased PtdIns-4,5-P_2 synthesis (140, 141). RhoA was also reported to increase PtdIns-4,5-P_2 production (138). Like PI3Ks, PIP5Ks may be regulated by both small G proteins and heterotrimeric G proteins. For example, a cholera toxin-sensitive G protein was found to regulate a 5-kinase activity in rat liver membranes (142). The availability of cDNA clones for the different PIP5Ks should facilitate the mapping of regulatory interaction sites and the mutational analysis of these regions and their physiological relevance.

PIP4Ks

MOLECULAR BIOLOGY Based on the ability to phosphorylate commercial PtdIns-4-P to produce PtdIns-4,5-P_2, a second form of PIP-kinase was purified (98, 143) and cloned (129, 144). This enzyme was thought to be a PtdIns-4-P 5-kinase and was named type IIα PIP5K. However, as discussed below, this PIP-kinase is actually a PtdIns-5-P 4-kinase (PIP4Kα). A second highly related gene (78% identical) has also been cloned (132). Previously termed type IIβ PIP5K, this enzyme is designated PIP4Kβ herein. Both cDNAs encode 47-kDa proteins whose kinase domains contain proline-rich sequences in contexts that match the consensus for binding to SH3 domains (Figure 4). The PIP4Ks are about 35% identical to the PIP5Ks in their kinase domains but are dissimilar in their amino- and carboxyterminal extensions (Figure 4). PIP4Ks also share homology with Mss4 and Fab1, and, like PIP5Ks, are more closely related to Mss4 (43%) than to Fab1 (28%) (131).

ACTIVITY AND REGULATION Recent work from our laboratory has established that in the presence of commercial PtdIns-4-P and [γ-^{32}P]-labeled ATP, the PIP4Kα produces PtdIns-4,5-P_2 radiolabeled at the 4′ position rather than at the 5′ position (34). This paradoxical observation is readily explained by the presence of contaminating PtdIns-5-P in the PtdIns-4-P used as substrate. Many commercial preparations of PtdIns-4-P are isolated from brain lipid extracts, so they also contain significant amounts of PtdIns-5-P, which was only recently found to exist in vivo (34) and is difficult to separate from PtdIns-4-P. The failure to phosphorylate the 5′ position of PtdIns-4-P may explain why red blood cell membranes could not be significantly phosphorylated by this enzyme (143); presumably, red cell membranes contain little PtdIns-5-P. Through the use of pure preparations of PtdIns-4-P and PtdIns-5-P, it has been found that PIP4Kα and β can phosphorylate only the latter (34; L Rameh, personal communication). Thus, these enzymes are PtdIns-5-P 4-kinases.

PIP4Kα also phosphorylates the 4′-OH of PtdIns-3-P in vitro (34, 133), although PtdIns-5-P is a better substrate (34). These enzymes probably account

for the PtdIns-3-P 4-kinase activity that was purified from platelets (145) and erythrocytes (97) using PtdIns-3-P as a substrate. PIP4Ks do not synthesize PtdIns-3,4,5-P_3 when PtdIns-3,4-P_2 is presented as a substrate (133), which strengthens the argument that these enzymes are not 5-kinases. In addition, they do not significantly phosphorylate PtdIns (L Rameh, personal communication). In view of their ability to phosphorylate the 4′ position of both PtdIns-3-P and PtdIns-5-P, we refer to these enzymes not as PtdIns-5-P 4-kinases but by the more general term PIP4K (Table 1) (34).

Little is known about the regulation of PIP4K activity. In vitro activity is inhibited by PtdIns-4,5-P_2 and by heparin. PIP4Kβ (previously termed type IIβ PIP5K) associates directly with the p55 subunit of the tumor necrosis factor (TNF) receptor, and indirect evidence suggests that its activity increases following TNF treatment of cells (132). An activity that converts PtdIns-3-P to PtdIns-3,4-P_2 is stimulated by an integrin-mediated pathway in platelets (145a). It is possible that the proline-rich motifs within the kinase domains of PIP4Ks mediate binding to proteins containing SH3 domains. Because PtdIns-3,4-P_2 is an important second messenger that participates in the activation of Akt protein kinase and certain PKCs (3), the PIP4Ks may play a role upstream of these kinases (145a).

PHOSPHOINOSITIDE SYNTHESIS: A CURRENT MODEL

Based on data discussed in this review, a model can be proposed for the synthesis of each of the known phosphoinositides. Although PtdIns has free hydroxyls at positions 2–6, only positions 3–5 are known to be phosphorylated in vivo (Figure 1). Figure 5 shows the phosphorylation steps reported to be catalyzed by purified, heterologously expressed PtdIns kinases and phosphoinositide kinases in vitro. Some of these pathways have been shown to occur in vivo, based on pulse-labeling studies and steady-state analysis of lipid levels in cells overexpressing constitutively active or dominant negative forms of the enzymes. Confirmation that other pathways occur in vivo awaits similar investigations.

Three different monophosphorylated forms of PtdIns have been shown to exist in vivo (Figure 1*b*). PtdIns is converted to PtdIns-3-P by PI3Ks (Figure 5). In vitro, all known types of PI3K catalyze this reaction. However, it is thought that most of the PtdIns-3-P in cells is synthesized by class III PI3Ks. Synthesis of PtdIns-4-P is catalyzed by PtdIns4Ks (Figure 5). PtdIns-5-P was recently identified in cells, when chromatographic separation from PtdIns-4-P was achieved (34). It is not yet known whether a phosphoinositide kinase generates PtdIns-5-P in vivo or whether it is produced only by the action of lipid

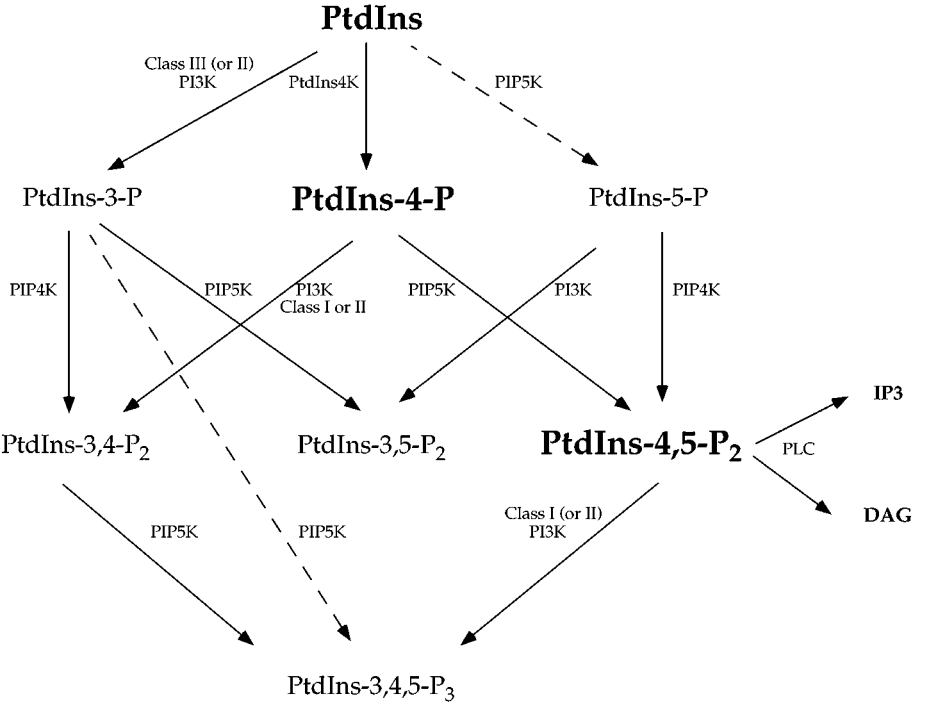


Figure 5 Flow chart of the established (*solid arrows*) and postulated (*broken arrows*) steps in phosphoinositide synthesis in mammalian cells. *Vertical arrows* indicate reactions catalyzed by phosphatidylinositol 4-kinases (PtdIns4Ks) or phosphatidylinositol-phosphate 4-kinases (PIP4Ks). *Diagonal-left arrows* are mediated by phosphoinositide 3-kinases (PI3Ks), *diagonal-right arrows* by phosphatidylinositol-phosphate 5-kinases (PIP5Ks). As stated in Figure 1, PtdIns-4-phosphate (PtdIns-4-P) and PtdIns-4,5-*bis*phosphate (PtdIns-4,5-P_2) (in larger type to emphasize abundance) together represent approximately 90% of the total phosphoinositides. PtdIns-3-phosphate (PtdIns-3-P) and PtdIns-5-phosphate (PtdIns-5-P) each represent approximately 2–5% of the total. PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 are barely detectable in quiescent cells and rise to about 1–3% of the total in stimulated cells. PtdIns-3,5-P_2 represents about 2% of phosphoinositides in fibroblasts. The levels of PtdIns, PtdIns-3-P, and PtdIns-4-P remain fairly constant in quiescent and activated cells, whereas relative amounts of PtdIns-3,4-P_2, PtdIns-4,5-P_2, and PtdIns-3,4,5-P_3 can change substantially under different conditions.

phosphatases. However, PIP5Ks phosphorylate the 5′-OH of PtdIns in vitro (Figure 5) (K Tolias, personal communication).

Three different *bis*phosphorylated forms of PtdIns have also been shown to exist in vivo (Figure 1*b*). PtdIns-4,5-P_2 is the predominant PtdIns *bis*phosphate in cells; its synthesis and hydrolysis have been studied in great detail. It is now clear that there are two pathways for synthesis of PtdIns-4,5-P_2: phosphorylation of PtdIns-4-P at the 5 position by PIP5Ks and of PtdIns-5-P at the 4

position by PIP4Ks (Figure 5). When cells are pulse labeled with ^{32}P, most of the radioactivity in PtdIns-4,5-P_2 is found at the 5 position, which suggests that the 5′ phosphate is usually added after the 4′-phosphate (146). This result is consistent with the greater abundance of PtdIns-4-P relative to PtdIns-5-P. It will be interesting to determine whether the ratio of labeled phosphates can be altered by overexpression of PIP5Ks versus PIP4Ks or in response to specific stimuli.

PtdIns-3,4-P_2 can be generated from PtdIns-4-P by class I and class II PI3Ks (Figure 5). In addition, it can be synthesized by phosphorylation of PtdIns-3-P by PIP4Kα (34, 133) (Figure 5). Although the PIP5Ks were reported to generate PtdIns-3,4-P_2 from PtdIns-3-P (133), a more recent study concluded that these enzymes preferentially phosphorylate the 5 position of PtdIns-3-P (K Tolias, personal communication). PtdIns-3,5-P_2 has been found in cells (137, 137a), and pulse-labeling studies show that the 5-phosphate is added last. This finding suggests that most of this lipid is produced by phosphorylation of the 5 position of PtdIns-3-P (137). However, this lipid can also be generated from PtdIns-5-P by PI3Ks in vitro (34).

It is well established that PtdIns-3,4,5-P_3 is synthesized by class I PI3Ks using PtdIns-4,5-P_2 as substrate (147) (Figure 5). In addition, the PIP5Ks can phosphorylate PtdIns-3,4-P_2 at the 5 position in vitro (133) (Figure 5). Interestingly, the PIP5Ks were also found to generate PtdIns-3,4,5-P_3 from the monophosphorylated PtdIns-3-P in a concerted reaction (133). There has been some controversy about the order of phosphate addition in the production of PtdIns-3,4,5-P_3 in vivo. Two early studies indicated that, in thrombin-stimulated platelets and platelet-derived growth factor (PDGF)-stimulated fibroblasts, the 5′ phosphate is added last, consistent with a role for a PtdIns-3,4-*bis*phosphate 5-kinase (148, 149). However, in later studies of formyl peptide-activated neutrophils (35), PDGF-stimulated fibroblasts (150), and thrombin-stimulated platelets (151), evidence supported addition of a 3′ phosphate to PtdIns-4,5-P_2. In view of the recent in vitro results, it would not be surprising if multiple pathways exist in vivo for the synthesis of PtdIns-3,4,5-P_3.

It is important to note that overall levels and local concentrations of the different phosphoinositides are also regulated by lipid phosphatases and by phospholipases. PtdIns-4,5-P_2 is the preferred substrate of phospholipase C (PLC) enzymes (Figure 5). Lipolysis of PtdIns-4,5-P_2 generates two second messengers with well-characterized actions: the lipid DAG, which activates some protein kinase C (PKC) isozymes, and the water-soluble inositol-1,4,5-*tris*phosphate, which promotes a rise in intracellular Ca^{2+} concentration (152). This process is sometimes referred to as the classical phosphoinositide-turnover pathway. Unlike PtdIns-4,5-P_2, the 3′-OH phosphoinositides, namely PtdIns-3-P,

PtdIns-3,4-P_2, and PtdIns-3,4,5-P_3, are poor substrates for PLCs (153). It is possible that these lipids are hydrolyzed by phospholipases that have yet to be discovered. Nevertheless, it is clear that 3′-OH phosphoinositides act directly as second messengers to regulate proteins at the membrane (3).

CONCLUDING REMARKS

The study of phosphoinositide kinases has progressed rapidly since PI3Ks were first cloned from yeast (85) and mammals (13). Molecular tools are now available to dissect the complex regulation of phosphoinositide synthesis. Several challenges remain. It will be important to determine the three-dimensional structures of all these enzymes. The protein kinase activity of PI3Ks and their detectable sequence homology with classical protein kinases suggest that their catalytic domains might fold similarly. It will be especially interesting to compare the PI3Ks and PtdIns4Ks with apparently unrelated families of lipid kinases, including the PIP4Ks and PIP5Ks and the TOR/ATM/DNA-PK group. These structural studies will be crucial for designing compounds to differentially modulate the activities of these enzymes for research and clinical applications. Another important task will be to compare expression of the different isoforms in different tissues as well as within subcellular compartments. Most studies of phosphoinositide levels have measured lipids in total cell extracts; a more precise understanding of lipid metabolism will require analysis of intracellular pools of the products of the different enzymes we have discussed here. Furthermore, investigation of phosphoinositide biology must be complemented by the study of lipid phosphatases. These enzymes may be just as important as lipid kinases in determining the balance of different phosphoinositides and lipid second messengers in the cell. These avenues of study are certain to increase our knowledge of how the cell generates and breaks down phosphoinositides to regulate downstream signaling.

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