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Probing phosphoinositide functions in signaling and membrane trafficking

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The inositol phospholipids (PIs) comprise a family of eight species with different combinations of phosphate groups arranged around the inositol ring. PIs are among the most versatile signaling molecules known, with key roles in receptor-mediated signal transduction, actin remodeling and membrane trafficking. Recent studies have identified effector proteins and specific lipid-binding domains through which PIs signal. These lipid-binding domains can be used as probes to further our understanding of the spatial and temporal control of individual PI species. New layers of complexity revealed by the use of such probes include the occurrence of PIs at intracellular locations, the identification of phosphatidylinositol signaling hotspots and the presence of non-membrane pools of PIs in cell nuclei.

Introduction

The inositol-containing glycerophospholipids, collectively known as phosphoinositides (inositol phospholipids; PIs), are among the most versatile of regulatory molecules, with strikingly diverse roles in cell signaling and vesicle-based transport mechanisms. This versatility arises from the chemistry of the *myo*-inositol moiety, which is attached to diacylglycerol (DAG) via a di-ester phosphate at the D-1 position, leaving five free hydroxyls, three of which are phosphorylated in different combinations by lipid kinases. PIs that have been identified in eukaryotic cells, their biosynthesis and metabolic interconversions are illustrated in Figure 1. The enzymes involved in these pathways have been reviewed extensively elsewhere [1–6]. With the recent discovery of phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] in fission yeast [7], it now appears that most of the PIs in Figure 1 are conserved from yeast to man. Here, we focus on PI-binding proteins and, in particular, on the use of specific lipid-binding domains as probes for the quantitative temporal and spatial analysis of PIs in cells (Box 1).

The DAG moiety accounts for the fact that PIs are predominantly if not exclusively associated with cell membranes. By contrast, it is the exposed headgroups of PIs that bind to effector proteins and through which their signaling functions are realized. The structural diversity of these headgroups, the existence of effector domains with high affinity and selectivity for particular PI species and the non-uniform distribution of PIs among subcellular membranes are crucial for the fidelity of PI-dependent signaling mechanisms. If the number of PI species seems complex and confusing, the range of effector domains is even more dramatically diverse. They include pleckstrin homology (PH), phagocyte oxidase (PX), epsin N-terminal homology (ENTH) and Fab1p, YOTB, Vac1p and EEA1 (FYVE) domains, as well as an assortment of proteins that bind to PIs with varying degrees of specificity through small patches of basic amino acids [4,8]. These domains serve primarily to target their host proteins to specific cellular locations and, in some cases, perhaps, might more directly regulate protein function. Their use as probes depends upon a high degree of specificity for the target lipid, affinities that allow detection of the target at the levels that occur naturally in cell membranes and an understanding of secondary interactions that might restrict the distribution of the probe to a particular compartment, limiting its use for detection of the target at other sites. With the possible exception of PtdIns5*P* and PtdIns itself, suitable probes now exist for all of the PIs in Figure 1, implying that each of these lipids has one or more signaling roles. What follows considers how we can use and avoid abusing these probes to study the functions of individual PI species.

PtdIns(4,5)P_2: multiple functions and locations

The lipid phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] undoubtedly represents a focal point in PI-dependent signaling in both metabolic and functional terms. It is synthesized mainly by a diverse family of PtdIns4*P* 5-kinases and serves as the substrate for two powerful receptor-regulated signal-generating enzymes. PI-phospholipase C (PI-PLC) cleaves PtdIns(4,5)P_2, simultaneously producing two second messengers, DAG and inositol (1,4,5)-trisphosphate [15]. Type I PI 3-kinases [1], on the other hand, convert PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3, of which more later. If this wasn't enough, PtdIns(4,5)P_2 itself can bind to an increasing number of effector domains, through which it appears to be a crucial regulator of: actin polymerization and anchorage to plasma membranes and vesicular structures; assembly/disassembly of vesicular coats; invagination and scission of endocytic vesicles; regulated secretion; the turnover of focal adhesion complexes and several types of plasma membrane K^+ channel [2,3,16–21].

Many of the proteins that bind to PtdIns(4,5)P_2 physiologically appear to do so through clusters of basic

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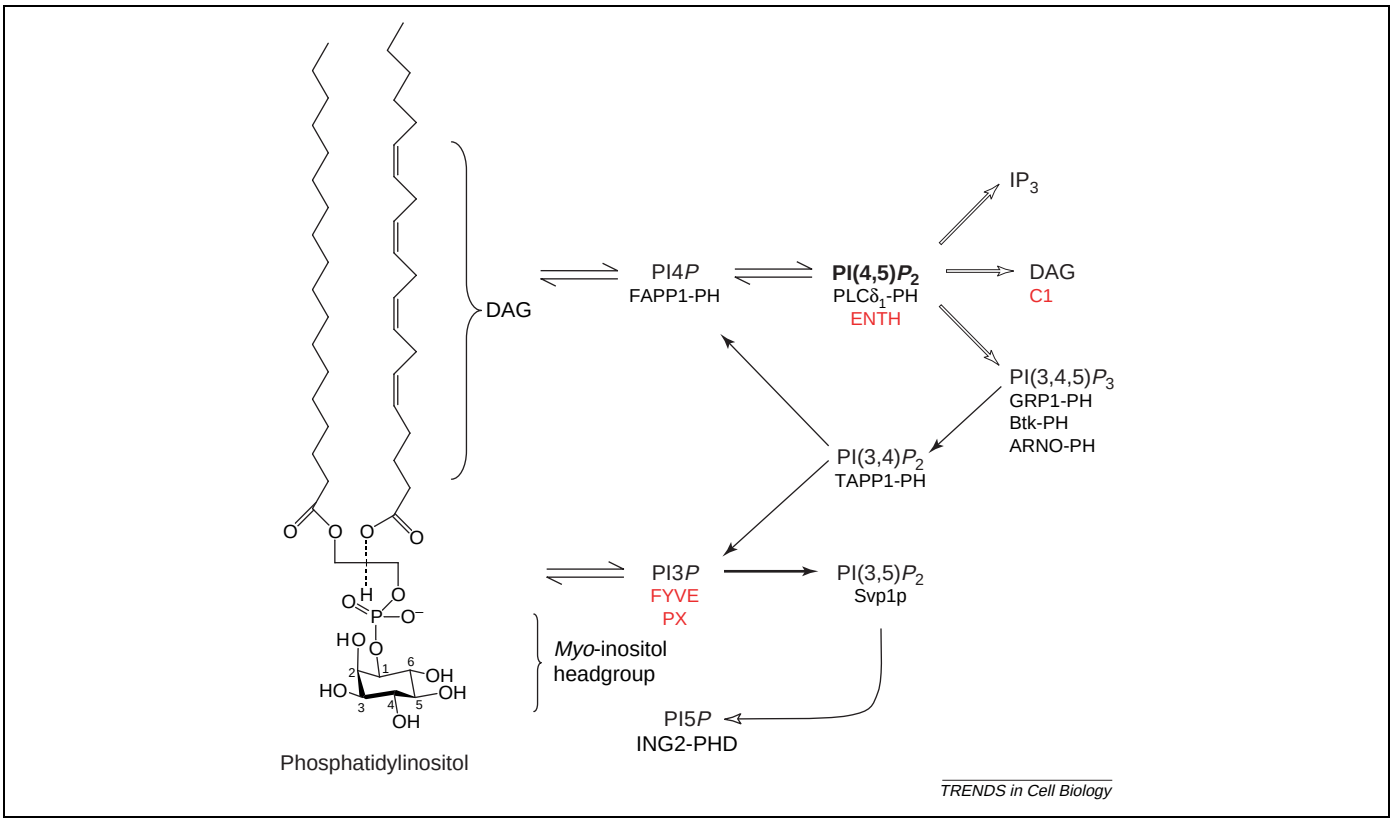

Figure 1. Biosynthesis and metabolism of phosphoinositide signals. Phosphatidylinositol (PtdIns) is the precursor of all the phosphoinositides (PIs) shown. Most of the naturally occurring phosphatidylinositol has a saturated fatty acid esterified at the 1-position of glycerol, with a polyunsaturated fatty acid at the 2-position (typically stearic acid and arachidonic acid, respectively, as illustrated). It is generally assumed that all PtdIns species share this composition, which is likely to have a bearing on whether PIs are components of lipid rafts. The PIs shown differ with respect to the number and distribution of mono-ester phosphate groups around the inositol ring and are interconverted by kinase and phosphatase enzymes that comprise 14 distinct enzyme families. Protein domains that have been shown to interact with a particular PtdIns species are indicated in red, while specific PI-binding proteins and the relevant domains they harbour are indicated in black.

residues against a relatively hydrophobic background but have little else in common and might not share a common structural fold. By contrast, three distinct domains have been shown to bind to this lipid, with varying degrees of specificity. These include proteins containing ENTH and AP180 N-terminal homology (ANTH) and PH domains [3,22]. Of these modules, the PH domain of PLCδ_1 has been extensively characterized and utilized as a probe to study PtdIns(4,5)P_2 dynamics in cells.

PH domains are protein modules of ~120 amino acids, and there are ~250 representatives within the human genome. While many PH domains bind to PIs, they often do so with relatively low affinity and/or specificity. PH PLCδ_1 is an exception in that it binds to PtdIns(4,5)P_2 and its headgroup, Ins(1,4,5)P_3, sufficiently selectively to be used as a cellular probe for these compounds [23–25]. The function of these interactions is to target PLCδ_1 to plasma membranes enriched in the substrate lipid, with Ins(1,4,5)P_3 competing for this binding and hence acting as a feedback inhibitor of PLCδ_1 when Ins(1,4,5)P_3 levels are raised. Recently, it has been shown that, while the interaction with PtdIns(4,5)P_2 appears essential for plasma membrane binding of PH PLCδ_1, this might also require additional interactions involving the C-terminal α helix and a short loop between the β_6 and β_7 sheets [26].

Green fluorescent protein (GFP)-tagged PH-PLCδ_1 labels predominantly the plasma membrane when analysed by confocal microscopy. The stimulation of PLC-coupled receptors is usually accompanied by a pronounced translocation of this membrane fluorescence to the cytosol. Several studies have concluded that the agonist-dependent translocation of PH-PLCδ_1 is due to increased cytosolic Ins(1,4,5)P_3 and not the decline of plasma membrane PtdIns(4,5)P_2 [24,25]. However, under circumstances where the intensity of stimulation leads to a significant fall in the concentration of PtdIns(4,5)P_2, it seems likely that this must also contribute to the cytosolic redistribution of the probe.

A potential limitation of studies with live cells is that overexpressed PI-binding proteins could sequester PtdIns(4,5)P_2 from its endogenous binding partners. This limitation can be overcome in experiments using the tagged PH domain on fixed cells. In this approach, cells are either permeabilized or sectioned to allow access of the immunological detection system. Using the on-section method adapted for electron microscopy, it has been possible to study the distribution of PtdIns(4,5)P_2 at high resolution (see Box 2). These studies revealed a major pool of PtdIns(4,5)P_2 in plasma membranes, and smaller pools in intracellular membranes, including Golgi, endosomes and endoplasmic reticulum [27]. PtdIns(4,5)P_2 was also found in the nuclear matrix, where it was associated not with membranes but with dense regions of heterochromatin, in agreement with studies using antibodies against PtdIns(4,5)P_2 [28] (see Box 3 for a discussion of nuclear inositol lipids). An important advantage of the

Box 1. Methods for determining PtdIns/protein binding affinities and specificities

Ligand binding specificity and affinity are key determinants of the suitability of any probe for analysing the cellular content, spatial distribution and dynamics of an individual phosphoinositide species (Table I).

Table I

Lipid/protein overlay ('Fat blot')

A range of lipids is spotted onto nitrocellulose, soaked in buffer containing the protein of interest, washed and absorbed protein detected by enhanced chemiluminescence [9].

Advantages	Disadvantages
Quick, easy technique familiar for non-specialist laboratories	Purely qualitative
Many lipid species analysed in parallel	Prone to both false positives and negative
	Must be used in conjunction with a quantitative approach

Pull down assays

Several variations on these, e.g. sucrose-loaded unilamellar lipid vesicles can be rapidly centrifuged for analysis of bound proteins by western blot. Alternatively, tagged proteins can be pulled down on agarose beads and absorbed lipid vesicles detected by using radiolabeled carrier lipids [10–12]

Advantages	Disadvantages
Quantitative	Slow separation of bound and free ligand means that low-affinity interactions will be underestimated
Vesicle composition can be varied to resemble cellular membranes	

Surface plasmon resonance (BIACore)

Several BIACore chips can be employed with protein attached when the adsorption of large unilamellar vesicles containing the lipid of interest can be detected. Alternatively, with lipids adsorbed to a hydrophobic chip, the binding of protein in the mobile phase can be detected [9,13].

Advantages	Disadvantages
Potentially quantitative	The nature of adsorption of lipids to hydrophobic chips remains ill-characterized
Lipid composition can be varied	Instrumentation is expensive and binding kinetics often prove complex
Binding to equilibrium makes it suitable for detecting low- as well as high-affinity interactions	

Ligand displacement

Adaptation of methods introduced for high-throughput screening of PI 3-kinases. Uses a sensor complex in which the binding of a pleckstrin-homology (PH) domain to its cognate lipid immobilized on allophycocyanin generates a time-resolved fluorescence resonance energy transfer (TR-FRET) signal. Exogenous lipids with high affinity for the PH domain dissociate the sensor complex and reduce the TR-FRET signal [13,14].

Advantages	Disadvantages
Very quantitative	Optimal signals require the use of low levels of detergent
Large number of assays run in parallel	
Suitable for both soluble (e.g. inositol phosphates) and lipid/vesicular ligands	

Box 2. Ultrastructural localization of phosphoinositides on cryosections

A traditional approach to localize lipids is to express GFP-tagged constructs in living cells, but these may have dominant interference effects on labeling. In on-section labeling, the cells are fixed and sectioned without prior transfection. The sections are then decorated using a tagged version of the lipid-binding module, which is then immunologically detected for microscopy. In the EM version illustrated here (Figure I), localization is performed on thin cryosections, using electron-dense marker colloidal gold as the readout. The affinity labeling is performed close to zero degrees C to prevent extraction of phosphoinositides (PIs) from the sectioned membranes [27]. When combined with unbiased stereological quantitation of the gold signal [30], this approach can be used to localize PIs in cellular compartments [27,29]. Controls for specificity include: (i) competing-out binding using soluble phosphoinositide headgroups [27], (ii) physiological modulation of endogenous PIs before fixation [27,29] and (iii) point mutations that reduce or prevent PI binding [29]. On-section labeling can also be applied to fluorescence microscopy for quantitative detection of PIs as a biomarker in clinical samples [31].

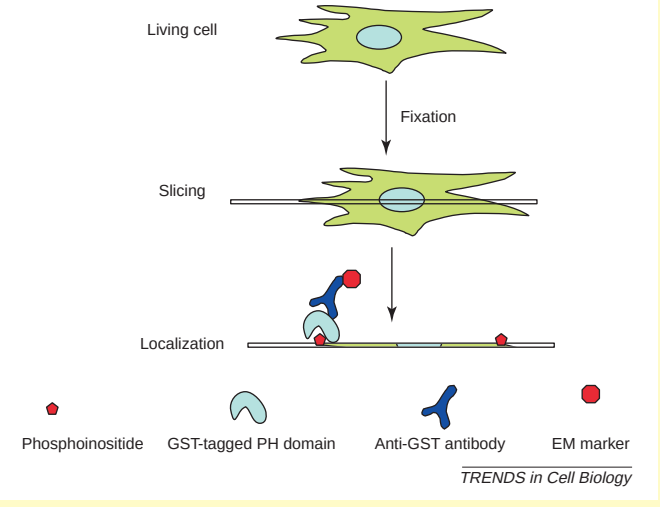

Figure I.

Box 3. Phosphoinositides in the nucleus: phospholipids, but not as we know them

Phosphoinositides (PIs) were first found to be components of highly purified nuclei more than 20 years ago [48]. It came as a big surprise, however, when it was discovered that PIs were present in nuclei that had been stripped of membrane structures [49]. Recently, probes for PtdIns(4,5)P_2 (see Box 2 and Ref. [48]) identified this lipid in the nuclear matrix within electron-dense structures, nuclear lamina and nuclear speckles. This raises important questions about the physicochemical form of these lipids in the absence of obvious membrane structures. As it can be detected with probes that interact primarily with the headgroup, it seems most likely that nuclear PtdIns(4,5)P_2 exists either in micellar or proteolipid complexes that envelop the hydrophobic fatty acids, leaving the headgroups exposed.

Functions of nuclear PIs

Nuclear PtdIns(4,5)P_2, as elsewhere in the cell, is likely to have multiple functions. It appears to be a substrate for PI-phospholipase C enzymes (PLCs; especially PLCβ_1) and hence might regulate DAG-sensitive protein kinase Cs. Although its role in intranuclear Ca^{2+} signaling is not clear, Ins(1,4,5)P_3, as it does in yeast, might instead serve as a substrate in the synthesis of several inositol polyphosphates implicated in the control of mRNA export and chromatin remodeling [50–52]. Nuclear PIs are also likely to be substrates for PI 3-kinases, but so far PtdIns3*P* is the only 3-PtdIns that has been detected directly [53]. The presence of PtdIns(3,4,5)P_3, however, is inferred by the occurrence of Type I PI 3-kinases and a novel regulatory GTPase (PIKE, PtdIns 3-kinase enhancer), one form of which is localized to nuclei [54]. As for nuclear PI-binding proteins, chromatin-associated ING2, through its plant homeodomain (PHD) finger, might be the first known functional binding partner for PtdIns5*P*, through which it might regulate p53-dependent responses to DNA damage [55].

on-section method is that it opens-up compartments and allows more even access of the labeling reagents to cellular structures. Furthermore, the quantitative evaluation of the particulate gold readout at the EM level allows dispersed, but weak-labeled, compartments to be detected (27,29,30).

A crucial question that can now be addressed is whether PtdIns(4,5)P_2 content is polarized in particular regions of membranes or uniformly distributed. The idea that lipid second messengers might be concentrated in membrane microdomains such as lipid rafts has arisen using mainly indirect approaches. In fact, as PIs commonly possess polyunsaturated fatty acids at the 2-position (see Figure 1), they seem unlikely raft components, a conclusion supported by ultrastructural analyses [32,33]. In a recent study, Huang *et al.* [34] applied ultrafast acquisition and super-resolution deconvolution microscopy to adipocytes expressing GFP-tagged PH-PLCδ_1. This approach revealed large-scale patches of plasma membrane that were enriched in PtdIns(4,5)P_2 beneath which were present clathrin-containing vesicular structures and dense concentrations of polymerized actin. These results provide evidence for the involvement of PtdIns(4,5)P_2 as a major determinant coordinately regulating actin polymerization and endocytic vesicle trafficking. Importantly, the PtdIns(4,5)P_2-rich patches observed in these studies were probably too large to correspond to rafts (with lateral dimensions of greater than several micrometers [32]). Ultrastuctural studies by on-section labeling (Box 2) also showed enrichment of PtdIns(4,5)P_2 in actin-rich lamellipodia-like structures [27]. It is important to consider how such apparently stable PtdIns(4,5)P_2-enriched loci might be maintained. As the rate of lateral diffusion of lipids within the bilayer is likely to be very fast compared with the turnover numbers of lipid-metabolizing enzymes. We suggest that barriers to diffusion would be needed to reduce the rate at which PtdIns(4,5)P_2 could escape from such 'hotspots' in addition to the specific targeting of synthetic enzymes to and/or degradative enzymes away from these zones. Such a diffusion barrier could be provided by the high concentration of PtdIns(4,5)P_2-binding proteins, associated with the regulation of actin turnover, surrounding such sites. These binding proteins would act like a fine sieve so that, at any one time, most of the ligand would be free (and hence detectable by the probe), but its progress through the sieve would be retarded. The combination of high-resolution microscopic techniques and the availability of selective binding probes can now begin to address whether PtdIns(4,5)P_2-enriched microdomains occur commonly and the mechanisms that support the polarized distribution of signaling lipids in cells.

PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2: lipids mediating PI 3-kinase-dependent signaling pathways

PtdIns(3,4,5)P_3 is synthesized by Type I PI 3-kinases (PI3Ks), a reaction that is reversed by the tumor-suppressor lipid phosphatase, PTEN (for 'phosphatase and tensin homolog deleted on chromosome ten'; see Figure 2). In quiescent cells, PtdIns(3,4,5)P_3 is present at low levels [~0.1% of the level of its precursor, PtdIns(4,5)P_2], but, upon stimulation of tyrosine kinase and some G-protein-coupled receptors, its concentration can increase by factors ranging from 2- to 100-fold. The sources of cellular phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P_2] are not completely established, but it probably arises mainly through the dephosphorylation of PtdIns(3,4,5)P_3 by 5-phosphatases such as SHIP (for 'SH2 domain containing inositol phosphatase') and SHIP2 [35] and it occurs in amounts similar to its precursor. Together, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 appear to account for the many and varied cellular functions of Type I PI 3-kinases, which include cell growth and proliferation, resistance to apoptosis, regulation of cytoskeleton dynamics, membrane trafficking and many of the metabolic responses to insulin [1,36]. These pervasive roles explain the intense interest in PI 3-kinase signaling pathways as a potentially rich source of targets for therapeutic intervention in cancer, type II diabetes, cardiac failure, inflammatory diseases and problems involving auto/hyper-immune responses.

PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 regulate a wide range of effector proteins primarily by targeting them to specific membrane locations via high affinity binding to one or more PH domains [9]. Because of the relatively low concentrations of PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 in cells, PH domains that bind to these lipids physiologically must display a high degree of specificity, especially by comparison with the much more abundant PtdIns(4,5)P_2. Several PH domains that display the requisite selectivity

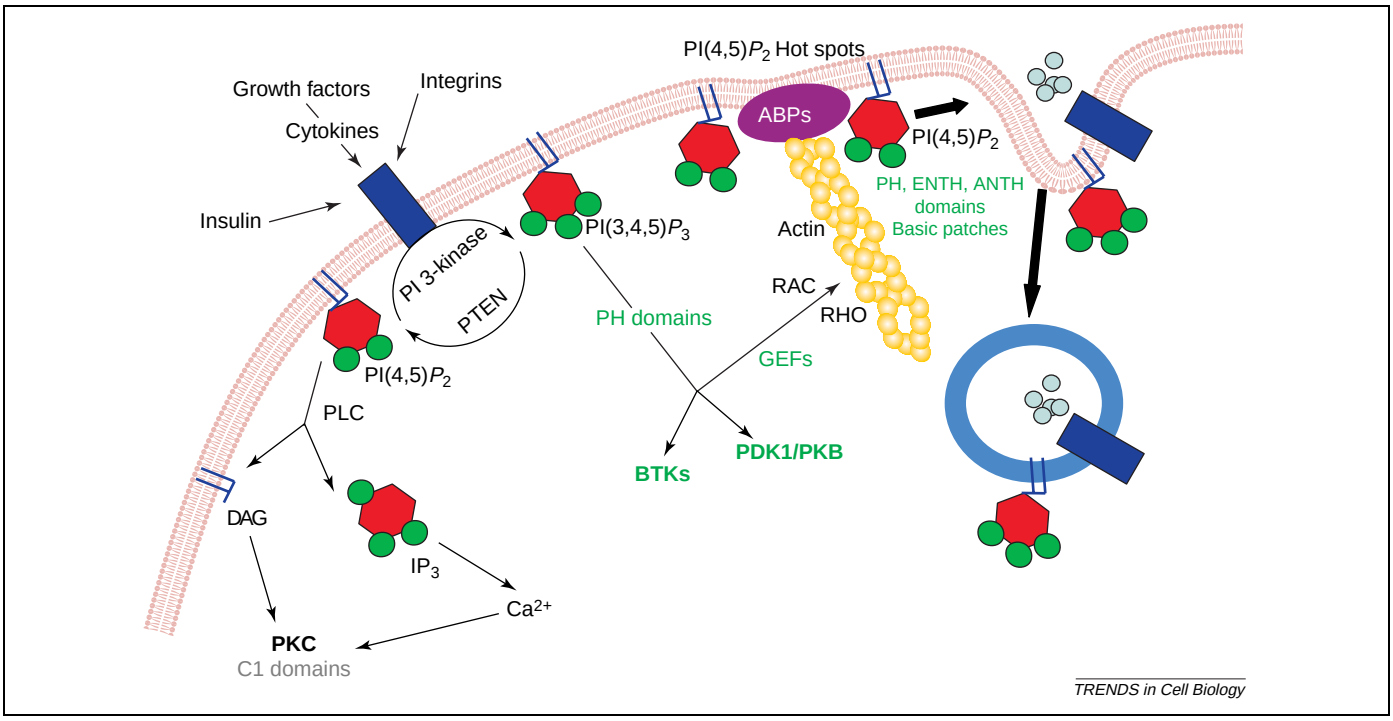

Figure 2. Lipid-binding domains mediating phosphoinositide functions at the plasma membrane. PtdIns(4,5)P_2 [PI(4,5)P_2] is the substrate for stimulus-dependent phospholipase C enzymes (PLCs) and type I phosphoinositide 3-kinases (PI 3-kinases) that generate three second messengers: soluble Ins(1,4,5)P_3 (IP_3) and membrane-associated diacylglycerol (DAG), and PtdIns(3,4,5)P_3 [PI(3,4,5)P_3]. PtdIns(4,5)P_2 [PI(4,5)P_2] also has a plethora of signaling roles. These include the regulation of actin polymerization and membrane anchorage, and endocytic vesicle trafficking. These functions are mediated through diverse protein domains (ENTH, ANTH and PH) and positively charged patches on structurally unrelated proteins. The diversity and relative abundance of actin-binding proteins capable of binding to PtdIns(4,5)P_2 might create an effective barrier to diffusion of this lipid away from F-actin-rich structures, possibly accounting for the occurrence of PtdIns(4,5)P_2 hotspots in such regions of the plasma membrane. PtdIns(3,4,5)P_3 binds to and regulates a wide range of proteins mediated through their PH domains, which account for their binding specificity towards lipids. Exemplified by Btk family members, serine/threonine kinases, such as PDK1 and PKB and GDP–GTP exchange factors (GEFs) for Rho-family G-proteins, which lead to regulation of actin dynamics. Agonist-stimulated endocytosis of growth-factor receptors leads to the internalization of signaling complexes, which retain activated PI 3-kinases and synthesize PtdIns(3,4,5)P_3 that accumulates in components of the endomembrane system.

have now been characterized and provide extremely valuable tools for studying the temporal and spatial distribution of PI 3-kinase signaling in cells. A problem with many of the PH domains that are in use for this purpose, for example the PH domain of protein kinase B/Akt, is that they bind to both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 [37]. The PH domains of Bruton's tyrosine kinase and several members of the general receptor for phosphoinositides 1 (Grp1) family (e.g. Grp1 itself, Arf nucleotide binding site opener, ARNO, and cytohesin 1), however, are highly selective for PtdIns(3,4,5)P_3. Recent crystallographic studies of Grp1 and ARNO PH domains have established that the insertion of a single glycine residue in the β_1/β_2 loops of these domains is sufficient to convert them from being monospecific to being dual binders of both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 [38].

When expressed in quiescent cells, GFP-tagged PtdIns(3,4,5)P_3-specific PH domains are mainly cytosolic, but they translocate primarily to plasma membranes in response to stimuli that activate PI 3-kinases [3,39,40]. This type of approach has been used to show the highly polarized accumulation of PtdIns(3,4,5)P_3 at the leading edge of *Dictyostelium* during chemotaxis towards a source of cyclic AMP [41]. A more sophisticated approach was used by Sato and colleagues [42] who developed a targetable probe in which Grp1–PH was fused between cyan and yellow fluorescent proteins. PtdIns(3,4,5)P_3 binding caused a conformational rearrangement that enhanced the fluorescence resonance energy transfer (FRET) signal between CFP and YFP. This probe was targeted to plasma membranes by inserting a CAAX motif and to endomembranes by mutation of Cys181 of the CAAX motif to a serine. In response to platelet-derived growth factor (PDGF), these probes detected rapid production of PtdIns(3,4,5)P_3 in plasma membranes, followed by its accumulation in endomembranes within ~1 min. Further experiments established that PtdIns(3,4,5)P_3 was produced *in situ* in endomembranes and required clathrin-mediated endocytosis of the PDGF receptor. Hence, there now exist probes that allow a high degree of temporal resolution of PI 3-kinase signaling in distinct subcellular compartments.

In addition to PH domains, monoclonal antibodies are available that interact with PtdIns(3,4,5)P_3, but which appear to detect principally cytosolic components in labeling experiments with fixed cells [43]. As the antibodies were raised against protein conjugates of the lipid headgroup, it might be that they are detecting either protein-bound lipid or indeed the headgroup itself, Ins(1,3,4,5)P_4.

Currently, the only well-characterized PtdIns(3,4)P_2-specific binding protein is the C-terminal PH domain of TAPP1 (for: 'tandem PH-domain-containing protein 1) [9,44]. TAPP1 might function as part of a feedback loop to downregulate tyrosine kinase signaling. It binds to the first of five PDZ domains of protein tyrosine phosphatase

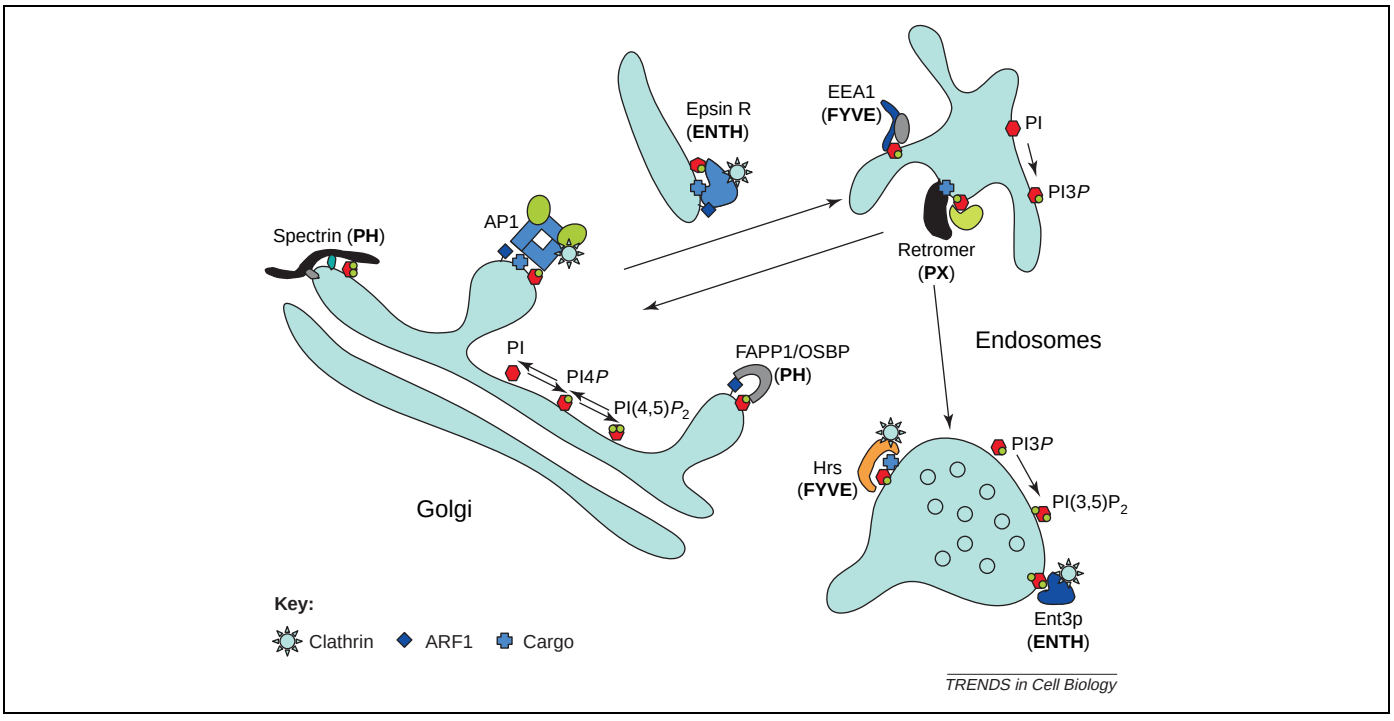

Figure 3. Subcellular distribution of phosphoinositides and localization of trafficking machinery in the Golgi complex and endosomal system. PtdIns4*P* (PI4*P*) and PtdIns3*P* (PI3*P*) are characteristic phosphoinositides of the Golgi and endosomal system, respectively. At these sites, accurate targeting of trafficking machinery is dependent on both lipid and protein–protein interactions (for example with small GTPases, cargo or coat proteins). In each case, the protein/protein complex is followed by the relevant lipid-binding domain in parenthesis. At the Golgi, PI4*P* is generated from phosphatidylinositol (PtdIns, PI) by PtdIns 4-kinases and acts as both a substrate for PtdIns(4,5)P_2 [PI(4,5)P_2] and a localizor of the budding machinery. For *trans*-Golgi localization, PtdIns4*P*-binding proteins (FAPP1, OSBP and AP1) engage with at least two components, PtdIns4*P* and the small GTPase ARF, and, in the case of AP1, also with cargo and clathrin. AP1 might act sequentially with GGA proteins in trafficking between the TGN and endosome, but the precise details of the trafficking steps are unclear. FAPP1 regulates Golgi export en route to the plasma membrane. The Golgi-localized lipid phosphatases driving hydrolysis of PtdIns(4,5)P_2 and PtdIns4*P* are not shown. In endosomes, PtdIns3*P* is synthesized through the action of the Vps34p and PtdIns(3,5)P_2 on late endosomes by mammalian PIKfyve/yeast Fab1p. PtdIns3*P*-binding domains targeting trafficking machinery to endosomes include FYVE, PX and ENTH (epsin N-terminal homology domain) domains. Initial weak membrane association by binding to PtdIns3*P* is strengthened by membrane insertion (FYVE, PX domains, ENTH) or by association with coat/tether proteins [retromer (PX); EEA1/rab5 (FYVE)]. The PtdIns3*P*-binding FYVE domain of Hrs sorts ubiquitinated cargo into clathrin-coated regions before uptake into multivesicular bodies, while the ENTH-domain-containing Ent3p is a clathrin binding effector of PtdIns(3,5)P_2 needed for a similar internalization step [together with another PtdIns(3,5)P_2-specific protein Ent5, Ent3 and the ubiquitin-binding protein Vps27p are required for sorting into the multivesicular body]. Another ENTH-domain-containing protein, Epsin R, mediates retrograde endosome–TGN traffic. It binds to PtdIns4*P* through its ENTH domain and its hydrophobic α-helix might be used to insert between lipid headgroups and induce membrane curvature. Epsin R can also interact with AP1 (PtdIns4*P* binder), clathrin and the SNARE protein Vti1b.

L1 (PTPL1) through its C-terminal tail and maintains the cytosolic pool of this phosphatase in quiescent cells. Upon stimulation of PI 3-kinase signaling, the increase in PtdIns(3,4)P_2 causes translocation of the TAPP1–PTPL1 complex to plasma membranes, where presumably it functions to reverse the actions of activated tyrosine kinases [45]. GFP-tagged TAPP1–PH is therefore a suitable probe for the detection of PtdIns(3,4)P_2 within cells. Recently, a GST-tagged TAPP1 PH has been utilized to map the ultrastructural distribution of its cognate lipid ligand by immunoelectron microscopy, as described in Box 2. These studies revealed the presence of PtdIns(3,4)P_2 in the endoplasmic reticulum and multivesicular endosomes, as well as in the plasma membrane [29]. Only the plasma membrane and endosomal pools were sensitive to the expression of PTEN, implying segregated metabolism and functions for these distinct pools of this signaling lipid.

The high degree of selectivity of certain PH domains for PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 has prompted their development as versatile tools in a range of assay procedures. These assays rely on the generation of a sensor complex involving the PH domain and a synthetic biotinylated version of the target lipid. The presence of exogenous lipid, produced for example in a PI 3-kinase assay, or present as a component of a tissue extract, can be quantitatively detected as it competes with the biotinylated lipid within the sensor complex [46]. These methods are proving extremely valuable in high-throughput screening of PI 3-kinases and as nonradioactive assays of signaling lipids in the tissues of transgenic animals carrying mutations of PI 3-kinase signaling components [47]. With the range of specific lipid-binding domains currently available, these methods should be adaptable specifically to the detection of most of the lipids depicted in Figure 1.

PtdIns4*P*, PtdIns3*P* and PtdIns(3,5)P_2: phosphoinositides associated predominantly with intracellular membranes

Research into the roles of PIs on intracellular membranes has lagged well behind that of PIs found at the cell surface. PtdIns(4,5)P_2, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are rarely found intracellularly, but a large body of evidence now implicates monophosphorylated PIs in the functions of intracellular organelles, in particular, membrane traffic [3,56]. The two principal lipids of interest are phosphatidylinositol 3-phosphate (PtdIns3*P*) and phosphatidylinositol 4-phosphate (PtdIns4*P*), of which the latter is the more abundant in cells and present at levels similar to those of PtdIns(4,5)P_2. These lipids target cytoplasmic

protein complexes to specific membranes, with PtdIns4*P* concentrated in the Golgi and PtdIns3*P* in the endosome system (Figure 3). Four principle PI-binding domains have been implicated in localizing functional proteins to these compartments, including PH domains (mainly Golgi localized), ENTH domains, found in Golgi and endosomes, and FYVE and PX domains involved in targeting to endosomes [8]. A major feature is their involvement in membrane trafficking and in the recruitment and turnover of cytoplasmic coats required for tubule and vesicle production [3,56]. Interestingly, a lipid interaction on the Golgi or endosomes is often not the sole determinant of membrane association, and other factors, usually proteins, are required [3]. This has two advantages for vesicle coat assembly/disassembly. First multiple low-affinity interactions facilitate dynamic interactions over the very small time scales needed for rapid coated-vesicle assembly, budding and coat disassembly reactions and, second, they allow lipid signals to be proof-read by a second or even third protein signal to target specifically to a small focus on a budding membrane [57]. Recruitment to the *trans*-Golgi network (TGN) is a good example.

The clathrin adaptor protein AP1 binds to PtdIns4*P* (although not through one of the recognized phospholipid-binding domains), but association with the membrane requires the small GTPase ARF [58]. AP1 also has to bind to cargo destined for transport in addition to its role in recruiting the clathrin coat. It is likely that AP1 dissociates from the membrane after budding, but presumably, as each one of these binding sites is removed, the binding becomes weaker and the complex falls apart. A second adaptor for clathrin-coated structures in the TGN region has been identified called Epsin R, and this contains an ENTH domain that is again selective for PtdIns4*P* [59]. Multiple interactions include an expected ARF1 interaction [60] and association with a SNARE protein cargo [61], and Epsin R appears to cooperate with AP1 to form clathrin-coated structures. Epsin R might function in retrograde trafficking from endosomes to the TGN [62]. Another multiple interaction mechanism has been observed for two proteins, FAPP1 and oxysterol-binding protein (OSBP), known to bind to PtdIns4*P* through their PH domains. FAPP1 was identified as a PtdIns4*P*-binding protein that controls traffic from the TGN to the cell surface and also binds to ARF1, which itself can promote recruitment of a PtdIns 4-kinase that could generate the FAPP1 ligand, PtdIns4*P* [63]. The interaction with ARF is mediated through the PH domain (63), and several other reports suggest that PH domains themselves can interact with proteins as well as with phosphoinositides [64–66]. Coat and cargo interactions have not so far been described for FAPP1. The functions and binding partners of PtdIns4*P* and PtdIns3*P* are illustrated in Figure 3.

So, are any of these lipid-binding domains useful as probes for PtdIns4*P*? When expressed in cells, GFP-tagged AP1 and FAPP1 show localization that is dependent on PtdIns4*P*, but the protein–protein interactions confound the unbiased detection of PtdIns4*P*. One solution might be to use on-section labeling because protein–protein interactions have a good chance of being disrupted by extensive aldehyde crosslinking, although careful controls would be required. One such control is to employ a mutant lipid-binding domain that no longer binds to the target phosphoinositide [27] or to make mutants that abrogate specifically the protein–protein interaction (Box 2).

Targeting to the endosomal system, rich in PtdIns3*P* generated from PtdIns by type III PI 3-kinase (yeast Vps34p and its mammalian homolog), has a decidedly different flavor. Here, two additional lipid-binding domains play a role in targeting both coat and tethering proteins. FYVE domains bind to PtdIns3*P* specifically and target several proteins to endosomal locations. Interestingly, the FYVE domain has rather limited affinity, explained by a relative lack of high-affinity hydrogen bonds being made with the PtdIns headgroup [8]. Affinity can be increased by nonpolar side-chains of a membrane insertion loop inside the membrane and/or by dimerization of FYVE domains. The latter is reflected in the need for using dimers of FYVE domains to label PtdIns3*P* in cells [53,67], although dimerization might not be required for FYVE domain proteins with higher affinity for membranes [68]. Additional interactions with coat, cargo and accessory proteins again ensure the precise localization, as in EEA-1 with Rab5 and Hrs with clathrin and ubiquitinated cargo destined for uptake into multivesicular endosomes.

Some PX domains also interact with PtdIns3*P* and are involved in protein sorting and vesicle coat assembly but, again, despite high selectivity, have a rather limited affinity for the lipid alone [3,8]. The affinity can be increased by insertion of a membrane association loop or by multiple interactions within a cytoplasmic coat such as the retromer complex, which mediates endosome-to-Golgi retrieval [69,70] and increases affinity by multiple PX interactions combined with cargo binding [71].

So, can the endosomal binding domains be used as probes for PI localization? This application has been pioneered by Gillooly *et al.*, who have used an FYVE domain dimer to localize PtdIns3*P* both in cells and on EM sections [53,67]. An FYVE domain construct that allows dimerization to be induced by exogenous addition of a cell-penetrant crosslinker has also been used to good effect in the detection of endosomal PtdIns3*P* [67]. Combining these approaches with the on-section labeling methods illustrated in Box 2 might be necessary to complete the picture in terms of the overall PtdIns3*P* content and distribution within cells.

Phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2] is the most recently identified phosphoinositide and is synthesized from PtdIns3*P* by Fab1p in *S. cerevisiae* and by an FYVE-domain-containing PtdIns kinase (PIKfyve) in mammalian cells [72,73]. In the latter case, the FYVE domain appears to target the enzyme to PtdIns3*P*-rich membranes (see above). The phenotype of *fab1*-deficient yeast is complex, implicating PtdIns(3,5)P_2 in vacuole-to-lysosome membrane trafficking, vacuole acidification, packaging of proteins in multivesicular bodies (MVBs) and growth at elevated temperatures, suggesting the probable involvement of functionally distinct PtdIns(3,5)P_2-binding proteins. This has now been confirmed with the identification of several effector proteins that appear to

bind to PtdIns(3,5)P_2 with the anticipated degree of specificity. These comprise Ent3p, Ent5p and Vps24p, which link Vps7p-mediated ubiquitin sorting into MVBs [74,75], Svp1p, which is required for membrane recycling from the vacuole [76], and sorting nexins that mediate transport from endosomes to the *trans*-Golgi [77]. As the yeast proteins do not account fully for the *fab1*Δ phenotype, it seems that additional PtdIns(3,5)P_2 effector proteins await discovery.

Surprisingly, these effector proteins interact with PtdIns(3,5)P_2 through a diverse set of PI-binding modules. For example, Vps24p and its mammalian homolog possess polybasic N-termini that do not resemble other known PI-binding domains, whereas Ent3p and Ent5p bind to PtdIns(3,5)P_2 through their ENTH domains [74,78]. Centaurin β_2, by contrast, has a PH domain that selectively binds to PtdIns(3,5)P_2 [9], while sorting nexins, such as SNX-1, utilize a PX domain for this purpose [77]. The most recently described PtdIns(3,5)P_2 effector protein, Svp1p, required for membrane recycling from the vacuole in yeast, is the first protein identified that binds to phosphoinositides through basic patches on a β-propeller structure [76].

Whether any of these modules represent suitable probes for the unbiased detection of PtdIns(3,5)P_2, however, is open to question. In the case of SNX-1, it is not clear whether PtdIns(3,5)P_2 or PtdIns3P is the physiological ligand. This protein also possesses a BAR (Bin/Amphiphysin/Rvs) domain that mediates dimerization and the detection of highly curved membrane surfaces [77]. So its cognate lipid ligands might only be detected with high affinity in the correct biophysical context. The full-length mammalian homolog of Vps24p binds to PtdIns(3,4)P_2 as well as to PtdIns(3,5)P_2 and, although it localizes to endosomes under certain conditions, this might involve additional hydrophobic interactions and/or oligomerization. Expression of the N-terminal half of this protein, which is responsible for phosphoinositide binding, exerts dominant-negative effects that clearly distort the labeled compartment, telling us much about the function of Vps24p but not about the global distribution of PtdIns(3,5)P_2. It might be that suitable probes could be developed as for PtdIns3P by enhancing the affinity of probes for PtdIns(3,5)P_2 by engineering suitable dimers or chimeras and combining live-cell and on-section labeling approaches.

Concluding remarks

The unique versatility of phosphoinositides as intracellular signals arises from four distinct aspects:

- The combinatorial actions of lipid kinases and phosphatases generates the range of molecules depicted in Figure 1, where many of the components are both precursors and products of signaling enzymes.
- A temporal aspect in which individual signals can be produced transiently and metabolized rapidly.
- A spatial aspect in which phosphoinositides are produced and maintained in distinct cellular locations and organelles.
- Finally, each PI can interact with a range of effector molecules, each of which must possess a PI-binding domain with an appropriate degree of specificity.

Future research will make increasing use of PI-binding domains and probes to study the spatial aspects of lipid signaling in increasing detail. 'Pure' PI-specific binding domains might, however, prove to be quite rare, while 'coincidence detection', in which the fidelity of protein targeting requires multiple, relatively low-affinity, interactions, appears especially important for many of the intracellular functions of phosphoinositides. The latter means that individual phosphoinositides can participate in the assembly of different signaling complexes in different locations. The usefulness of PI-binding domains as probes depends on understanding their lipid binding characteristics and any secondary interactions that might contribute to localization within cells. Even where secondary interactions are important, useful probes can be engineered, for example by making dimers of the lipid-binding moiety, and dominant interference effects can be avoided by on-section labeling techniques. With these points in mind, you might never again need to reach for the organic solvents or have a grasp of thin-layer chromatography to bring inositol phospholipids into your experimental repertoire.

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