Modular phosphoinositide-binding domains – their role in signalling and membrane trafficking

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The membrane phospholipid phosphatidylinositol is the precursor of a family of lipid second-messengers, known as phosphoinositides, which differ in the phosphorylation status of their inositol group. A major advance in understanding phosphoinositide signalling has been the identification of a number of highly conserved modular protein domains whose function appears to be to bind various phosphoinositides. Such 'cut and paste' modules are found in a diverse array of multidomain proteins and recruit their host protein to specific regions in cells via interactions with phosphoinositides. Here, with particular reference to proteins involved in membrane traffic pathways, we discuss recent advances in our understanding of phosphoinositide-binding domains.

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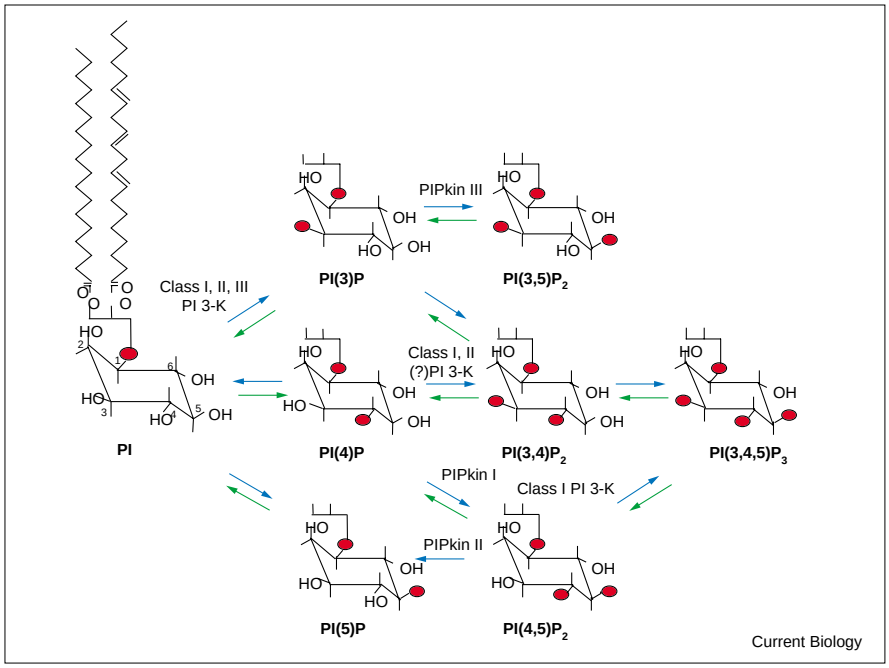
Introduction

The membrane phospholipid phosphatidylinositol is the precursor for a family of lipid second-messengers, known collectively as phosphoinositides, that differ solely in the phosphorylation status of their inositol head group (Figure 1) [1,2]. Phosphoinositides are ideally suited to function as spatially restricted membrane second messengers because: the synthesis and turnover of phosphoinositides from the relatively abundant phosphatidylinositol membrane precursor can be rapid and highly concentrated within discrete membrane micro domains; the ratio of phosphoinositide to binding partner can be relatively large, which makes it possible to target a large number of distinct proteins to a particular membrane without saturating the binding sites; structurally distinct phosphoinositides can activate distinct downstream effectors; and the potential for rapid, sequential interconversion between phosphorylated forms means that phosphoinositides can confer processivity to membrane signalling events.

A major advance in our understanding of phosphoinositide signalling has been the identification of a number of highly conserved modular protein domains that bind various phosphoinositides. To date, a number of distinct, phosphoinositide-binding motifs have been identified and characterised including the epsin amino-terminal homology (ENTH) domain, Fab1, YOTB, Vac1 and EEA1 (FYVE) domain, band 4.1, ezrin, radixin and moesin (FERM) domain, pleckstrin homology (PH) domain and more recently the phox homology (PX) domain [3]. Such 'cut and paste' modules are found in a diverse array of multidomain proteins and recruit their host protein to specific regions in cells via their interactions with phosphoinositides. They may also serve as allosteric regulators of enzyme activity and protein–protein interactions.

The binding of phosphoinositides to these domains is generally of low affinity and rapidly reversible which favors a highly plastic system. These features enable signalling proteins involved in phosphoinositide-mediated membrane association to undergo a sequence of random diffusions in the cytosol, interspersed with membrane binding and dissociation events [4], rather than to associate persistently with the membrane. Thus these signalling proteins are constantly sampling a large area of the membrane environment for the presence of a phosphoinositide-binding partner and any elevations in the mass of the partner lipid results in a local enrichment or translocation of the signalling protein. As specific signalling phosphoinositides can be readily produced and degraded by enzymatic activities,

Figure 1



Phosphoinositide metabolism. Phosphatidylinositol (PI), the basic building block for the intracellular inositol lipids in eukaryotic cells, consists of D-*myo*-inositol-1-phosphate liked via its phosphate group to almost exclusively 1-stearoyl, 2-arachidonoyl diacylglycerol – a fatty acyl composition that may contribute to an adequate packing of the phosphoinositides leading to efficient exposure of their inositol head group for interaction with cytosolic proteins. Unlike the head groups of other phospholipids, the inositol ring of phosphatidylinositol can be reversibly phosphorylated at one or a combination of the 3′, 4′ or 5′ positions. Phosphatidylinositol and its phosphorylated derivatives are collectively referred to as phosphoinositides. Phosphatidylinositol is the most abundant inositol lipid in mammalian cells under basal conditions, present at levels 10–20 times higher that those of phosphatidylinositol 4-monophosphate (PI(4)P) and phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), which are present in roughly equal amounts. Of the total singly phosphorylated phosphoinositides in cells, 90–96% is PI(4)P; phosphatidylinositol 3-monophosphate (PI(3)P) and phosphatidylinositol 5-monophosphate (PI(5)P) each make up about 2–5%. $PI(4,5)P_2$ is the most abundant of the doubly phosphorylated phosphatidylinositols (>99%); phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$) and phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$) each make up about 0.2% of the PIP_2. The levels of phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) vary enormously but can be comparable to those of $PI(3,4)P_2$ and $PI(3,5)P_2$.

lifetimes of phosphoinositide-binding partner complexes can be in the order of seconds to minutes.

Phosphoinositide-binding proteins are therefore a restless subclass of signalling proteins that spend a significant proportion of their time diffusing through the cytosol, ready to interact with their cognate phosphoinositide-binding partner if and when they meet it. Here, with particular reference to proteins involved in membrane traffic pathways, we discuss recent advances in our understanding of phosphoinositide-binding domains.

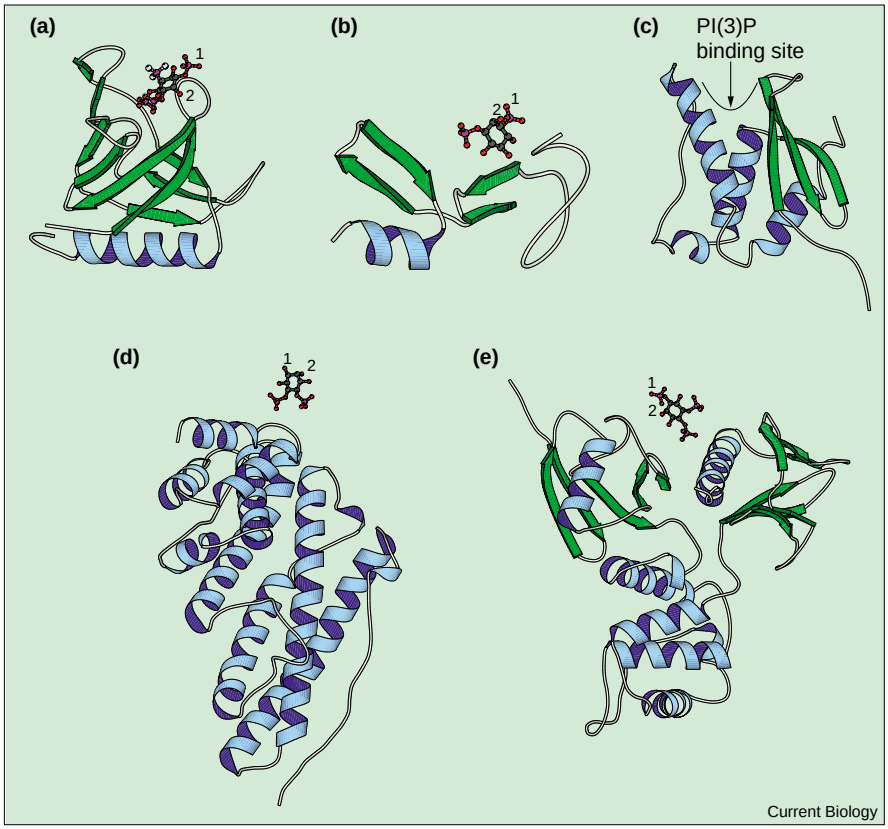
Pleckstrin homology domains

The recent first draft of the human genome sequence showed that PH domains are the eleventh most common domain in humans, appearing some 252 times in the deduced human proteome. PH domains are small β-sandwich protein modules of approximately 120 residues that occur once or, more rarely, several times in a protein sequence (reviewed in [5]; see Figure 2). Nearly all PH domain-containing proteins require membrane association for some aspect of their function and most if not all PH domains bind to phosphoinositides present in cell membranes although with different degrees of specificity and affinity. In 15–20% of cases phosphoinositide binding is strong and highly specific. Studies using green fluorescent protein (GFP) fusions have shown that phosphoinositide-binding by PH domains is sufficient for their independent, signal regulated targeting to cell membranes [3,6].

One of the first examples of such dynamic membrane targeting was described for the ARF6 exchange factor ARNO [7]. This cytosolic protein undergoes a rapid recruitment to the plasma membrane ($t_{½}$ approximately 30 seconds), following agonist activation of PI 3-kinase, *via* the specific interaction of its PH domain with the lipid product of PI 3-kinase activation, phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (Figure 3). In contrast the PH domain of phospholipase Cδ1 (PLCδ1), which specifically binds phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), constitutively targets this protein to the plasma membrane — an interaction that regulates the activity of the enzyme [8].

The ability of certain PH domains to interact specifically with a given phosphoinositide has recently been used to gain invaluable insight into the spatial and temporal organisation of phosphoinositide signalling events. Using the discovery that GFP and its variants can be used as expressed fluorescence tags to follow the subcellular localisation of signalling proteins in live cells, a number of groups have

Figure 2



Structure of phosphoinoisitide-binding modular domains. In the last year we have witnessed a dramatic increase in our appreciation of how specific modular binding domains have evolved to recognise their cognate phosphoinositide ligand. Depicted here are the backbone structures for **(a)** the PIP_3-binding PH domain from Grp1 in complex with inositol 1,3,4,5-tetrakisphosphate [99,100], **(b)** the PI(3)P-binding FYVE domain from EEA1 bound to inositol 1,3-bisphosphate [65], **(c)** the PI(3)P-binding PX domain from $p47^{phox}$ showing the predicted PI(3)P-binding sites as determined by NMR [75,101], **(d)** the PIP_2-binding domain from the clathrin assembly lymphoid myeloid leukemia protein (CALM) in complex with inositol 4,5-bisphosphate [33] and finally, **(e)** the PIP_2-binding FERM domain from radixin in complex with inositol 1,4,5-trisphosphate [102]. From comparing all of these structures it is immediately apparent that the structure of the phosphoinositide-binding site is not conserved between different types of phosphoinoisitide-binding domain (see also the recently solved crystal structure of the PX domain from $p40^{phox}$ bound to PI(3)P [103]).

tagged various PH domains to analyse stimulus-induced changes in specific phosphoinositides [6]. For example, a GFP conjugated PH domain from PLCδ1 has been used as a detector for $PI(4,5)P_2$ [8–16] whereas a corresponding GFP conjugated PH domain, found in ARNO and other specific PIP_3-binding PH domains, functions as a biosensor for PIP_3 [7,17–21]. The recent availability of these reagents, and similar tools based on other phosphoinositide-binding modules (see below), has allowed researchers to study phosphoinositide signalling in living cells. These studies have illustrated the highly dynamic and localised nature of phosphoinositide signalling.

Phosphatidylinositol 4,5-bisphosphate: a localised regulator of membrane events?

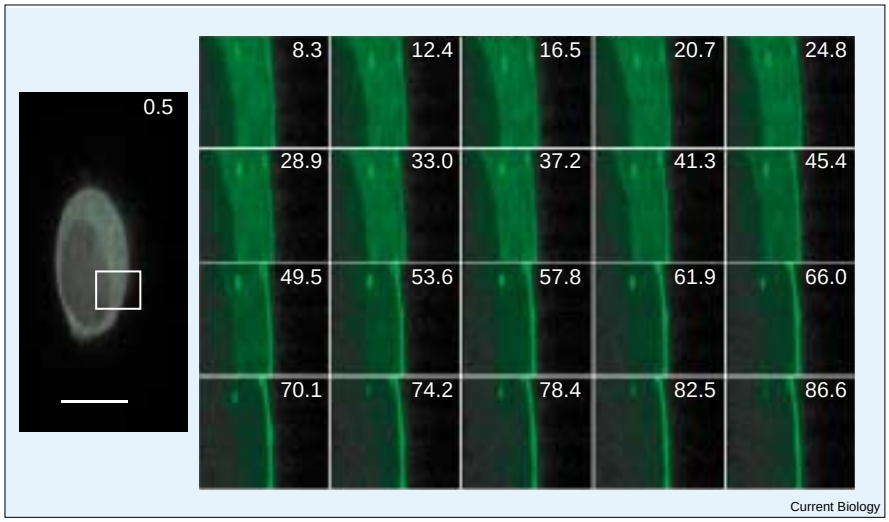
Using the $PI(4,5)P_2$ biosensor a number of recent studies have shown that $PI(4,5)P_2$ is mainly concentrated in the plasma membrane, with dynamic changes in its levels occurring at active sites of phagocytosis [12], actin-rich protrusions and membrane ruffles [13]. These observations, together with data from biochemical and immunocytochemical assays showing the presence of discontinuous, raft-like clustering of $PI(4,5)P_2$, support the idea that this lipid is not uniformly distributed on the inner leaflet of the plasma membrane but rather that it exists in gradients and regionally localised areas of the plasma membrane [22]. Such dynamic and highly localised elevation of $PI(4,5)P_2$ may result from the selective recruitment or activation of specific phosphoinositide kinases increasing *de novo* synthesis of $PI(4,5)P_2$. Alternatively, under resting conditions the pool of free $PI(4,5)P_2$ may be effectively sequestered by high affinity interactions with basic proteins or basic domains of specific proteins [23].

Inhibition of these interactions would provide a potential mechanism for rapidly releasing a local pool of $PI(4,5)P_2$ for interactions with other proteins. Such a strategy would not require the targeting or activation of phosphoinositide kinases, but would require the local regulation of the interaction between $PI(4,5)P_2$ and its tethering proteins [23]. Although our knowledge of the *in vivo* regulation of enzymes that regulate $PI(4,5)P_2$ synthesis is far from complete, recent results have begun to shed some light on these issues.

It appears that animal cells use two different routes of $PI(4,5)P_2$ synthesis (see Figure 1). One pathway, the 'classical PI cycle', involves the sequential phosphorylation of

Figure 3

Temporal dynamics of the epidermal growth factor-induced plasma membrane association of the PIP_3-binding protein ARNO. Time-lapse confocal imaging was performed on PC12 cells transiently transfected with a construct expressing GFP-tagged ARNO. 24 h after transfection cells were serum-starved for 2 h prior to imaging of a selected cell using an Ultra^{View} confocal microscope. Images were collected every 250 ms (for clarity only selected images are shown). Epidermal growth factor (100 ng/ml) was added immediately after collection of the 20.7 s image. Culturing and manipulation of transfected cells were as described in [7]. The complete series of images are presented as a time-lapse movie that can be seen online at http://images.cellpress.com/supmat/supmatin.htm.



phosphatidylinositol to phosphatidylinositol 4-monophosphate (PI(4)P) and PI(4,5)P_2 [1,2]. In the second pathway, only recently revealed, PI(4,5)P_2 is synthesised from phosphatidylinositol via the novel lipid phosphat-idylinositol 5-monophosphate (PI(5)P) [24]. The second step in each pathway is catalysed by homologous lipid kinases, termed type I and type II PIP kinases (Figure 1). Hence, type I PIP kinases phosphorylate PI(4)P to PI(4,5)P_2 and are PI(4)P 5-kinases [24–26], whereas type II PIP kinases generate PI(4,5)P_2 by phosphorylation of PI(5)P and are consequently PI(5)P 4-kinases [24,26].

Although type I and II PIP kinases share significant sequence identity they are not functionally redundant and mainly localise to distinct subcellular compartments. In mammalian cells, type I PIP kinases are found at the plasma membrane where they have been implicated in the regulation of actin rearrangements, secretion, and endocytosis [26]. In contrast, type II PIP kinases are principally localised to the cytosol, nucleus, endoplasmic reticulum, and the actin cytoskeleton [26].

The type I PIP kinase PI(4)P 5-kinase α is a highly regulated enzyme both allosterically and through membrane targeting. It has been shown that PI(4)P 5-kinase α is activated by the GTP-bound form of the small GTPases Rac, Rho and ARF6 as well as the product of phospholipase D activation, phosphatidic acid [27]. The regulation of PI(4)P 5-kinase α by both phosphatidic acid and ARF6–GTP suggests a potential mechanism for an explosive feed-forward regulatory loop for the acute, localised synthesis of PI(4,5)P_2. Given that PI(4,5)P_2 and activated ARF6 act synergistically to increase the activity of phospholipase D, an elevation in active ARF6 would induce an acute increase in phosphatidic acid which would itself stimulate PI(4)P 5-kinase α to produce more PI(4,5)P_2.

The feed-forward aspect of this loop stems from the ability of PI(4,5)P_2 to activate certain ARF6 exchange factors thereby increasing the GTP loading on ARF6, stimulating PI(4)P 5-kinase α, to produce more PI(4,5)P_2. Such a mechanism is further supported by the demonstration that ARF6, phospholipase D and PI(4)P 5-kinase α are recruited to active sites of phagosome formation and sites of cytoskeleton assembly at membrane ruffles [12,13,27–31]; these regions are rich in PI(4,5)P_2. Thus, mutual interactions between PI(4,5)P_2 and the proteins that regulate its synthesis probably result in a highly cooperative and dynamic regulation of PI(4,5)P_2 synthesis in restricted membrane domains.

Phosphatidylinositol 4,5-bisphosphate as a signal for membrane internalisation events

The concept of PI(4,5)P_2 as a spatially localised regulator of membrane events is clearly illustrated by its key role in clathrin-mediated endocytosis. This complex mechanical process is driven by the sequential action of a host of cellular proteins, many of which are recruited to the endocytic site through PI(4,5)P_2-binding modules [32]. Endocytosis can be broken down into four distinct steps, the first being the recruitment of clathrin to the endocytic site. This process can be reconstituted *in vitro* by combining purified clathrin, PI(4,5)P_2-containing liposomes and just one cofactor, the AP180 protein [33,34]. AP180 (and the related protein CALM) binds directly to clathrin but also contains a copy of the epsin amino-terminal homology (ENTH) domain [35]. The ENTH domain is a phosphoinositide-binding module that has recently been shown to

confer specific $PI(4,5)P_2$ binding to a number of proteins involved in endocytosis [32,36] (Figure 2). This fundamental observation is important as it suggests that $PI(4,5)P_2$ may act as a site selection signal for endocytic events.

As yet there is no direct evidence for localised concentrations of $PI(4,5)P_2$ at sites of endocytosis, however agents that disrupt lipid rafts have been shown to block clathrin-mediated endocytosis [37,38], suggesting that structured micro domains may be acting as endocytic seed sites. In this respect it is interesting to note that type IIα PI 4-kinase has been shown to localise to lipid rafts through a palmitoylation modification [39,40], suggesting, at least, that concentrations of the precursor of $PI(4,5)P_2$ exist in the plasma membrane. One prediction of this hypothesis is that site-directed mutagenesis of the palmitoylation site in type IIα PI 4-kinase would disrupt endocytosis.

Another important question is whether $PI(4,5)P_2$ acts solely as a passive marker for the entry site [41] or whether levels of $PI(4,5)P_2$ can be acutely regulated to govern endocytic rate. The epidermal growth factor (EGF) receptor has been shown to bind to type IIβ PI(5)P 4-kinase on activation [42,43], raising the possibility of local increases in $PI(4,5)P_2$ concentration in this case. EGF is known to stimulate endocytic rate; however, this has been attributed to activation of the Rab5 GTPase [44]. More definite evidence comes from recent studies of endocytosis in synaptic terminals. Here, postsynaptic activity was shown to stimulate $PI(4,5)P_2$ production on internalising endosomes at the presynaptic terminus through a nitric oxide dependent pathway [45]. In this case at least, it would seem that $PI(4,5)P_2$ levels can be used to control the rate of internalisation.

The subsequent steps of endocytosis also require $PI(4,5)P_2$: invagination of the plasma membrane to form a clathrin-coated pit is driven by recruitment of the AP-2 adaptor [33]. This heterotrimeric protein binds to the clathrin lattice through its β2-subunit and to $PI(4,5)P_2$ through an amino-terminal basic motif in its α-subunit [46]. In addition to contributing to the conformation of the clathrin-coated pit, AP-2 also binds to the tyrosine-based internalisation motif of certain receptors through its μ-subunit [47]. Following invagination, constriction and ligation of the neck of the coated pit occurs to generate an internalised coated vesicle. This process is mediated through recruitment of the GTPase dynamin which makes a number of interactions including direct contact with the membrane through its $PI(4,5)P_2$-binding PH domain [48,49].

During the final stage of endocytosis, the internalised vesicle looses its clathrin coat, producing a nascent early endosome. The uncoating reaction depends on the actions of synaptojanin-1, a phosphatidylinositide phosphatase that rapidly terminates the $PI(4,5)P_2$ signal, allowing release of the $PI(4,5)P_2$-dependent cofactors and so imparting directionality to the process of internalisation [50].

A number of other accessory factors with less well-defined roles are recruited to sites of clathrin-dependent endocytosis through $PI(4,5)P_2$-binding modules [32]. One intriguing example is the Huntingtin interacting-protein related-protein 1 (HipR1). HipR1 contains an amino-terminal ENTH domain and is tightly localised to clathrin-coated vesicles [51]. It also contains a carboxy-terminal actin-binding module, bringing us to the question of the function of actin in endocytosis.

Phosphoinositides play a fundamental role in regulating the actin cytoskeleton [52] and it would seem likely that the clustering of $PI(4,5)P_2$ during endocytosis would affect the local cytoskeletal framework. The actin cytoskeleton is required for several endocytic steps in yeast [53], and deletion of the yeast homologue of HipR1, Sla2p, causes endocytic defects [54]. In higher eukaryotes, the function of actin in endocytosis is less clear, however several proteins involved in endocytosis bind to F-actin directly, such as HipR1, or indirectly, such as dynamin [55]. One hypothesis is that local reorganisation of the cortical actin cytoskeleton, a mesh of polymerised actin lying directly under the plasma membrane, may be required to allow passage of the budding pit [55]. Certainly, the area directly surrounding clathrin-coated pits is depleted of polymerised actin [56].

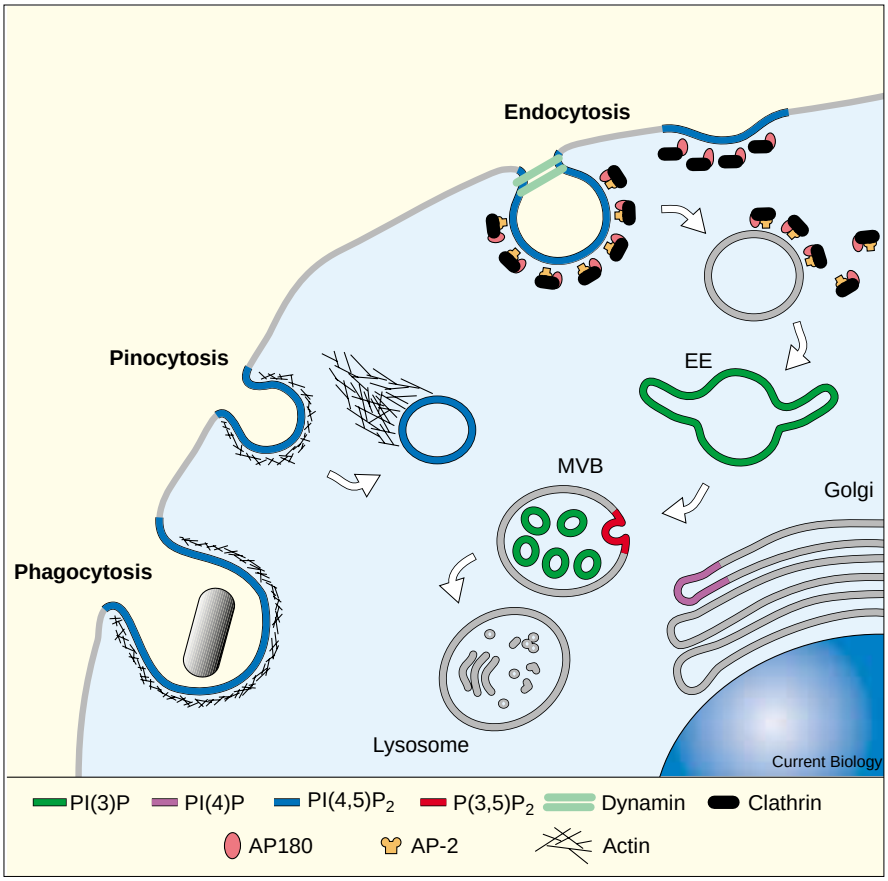
The actin cytoskeleton may also be involved in the spatial restriction of endocytosis. At neuromuscular junctions, a clearly visible barrier of polymerised actin defines highly concentrated sites of endocytosis adjacent to sites of neurotransmitter release [55]. These synaptic structures represent a specialised and highly efficient form of endocytosis, however, studies with actin depolymerising agents suggest that the actin cytoskeleton has a general role in maintaining restricted sites for endocytosis [56].

Perhaps the most surprising role for actin in endocytic traffic comes with the recent discovery of vesicle propulsion within the cell by short-lived 'comet tails' of polymerised actin [57]. This is a $PI(4,5)P_2$-dependent event that can be driven by overexpression of type I PI(4)P 5-kinase [58]. The function of the $PI(4,5)P_2$ is almost certainly to recruit and activate members of the Wiskott-Aldrich Syndrome protein (WASP) family, which in turn recruit and activate the actin branching complex Arp2/3 [58].

Despite the concentration of $PI(4,5)P_2$ in clathrin-coated vesicles, analysis of the identities of the vesicles propelled by actin comets suggests that this process has greater relevance to internalisation of cargo through clathrin-independent or macropinocytic routes [58]. It is possible that the clathrin-coat excludes assembly of polymerised actin,

Figure 4

Localization of phosphoinositides in membrane trafficking pathways. Work using GFP-based biosensors has revealed discrete localizations of phosphoinositides at sites of membrane traffic. PI(4,5)P_2 is seen to mark sites of membrane budding and fusion in processes like endocytosis and phagocytosis. PI(3)P marks early endosomes (EE) and internal structures in late multivesicular endosomes (MVB). The esoteric lipid PI(3,5)P_2 plays a role in sorting into these late endosomal structures. This snapshot of phosphoinositide localization illustrates their cellular function as markers of membrane events. This is only half of the story – the interconversions between phosphoinositides in membrane traffic are highly dynamic and impart processivity to trafficking pathways (see text for more details).



either physically or through sequestration of the PI(4,5)P_2 head groups. Certainly, later uncoated endosomal pathway components, such as multivesicular endosomes and lysosomes are capable of forming actin comets [59]. It has been proposed that these short-lived actin tails allow vesicles to explore their local environment and locate microtubule tracks. It is tempting to speculate that the extremely transitory nature of these structures is linked to rapid turnover of phosphoinositides on the vesicle surface and it will be interesting to test this hypothesis using GFP-linked biosensors.

Phosphatidylinositol 3-monophosphate in endosomal sorting – the role of FYVE domains

After internalisation, cell surface molecules, including receptors, are delivered to the peripheral early endosomes [60]. Early endosomes fuse not only with incoming vesicles from the surface, but also undergo homotypic fusion, resulting in a highly dynamic structure. Proteins in the early endosome are sorted for delivery to one of at least three endosomal destinations: the surface for rapid recycling; the lysosome via the late endosome; or the perinuclear recycling endosome (Figure 4).

A direct role for phosphoinositides in endosomal trafficking was originally demonstrated by genetic studies of protein transport to the yeast vacuole, the equivalent of the mammalian lysosome. More than forty vacuolar protein-sorting (*VPS*) genes are required for transport from the Golgi apparatus to the vacuole. One of these genes, *VPS34*, was found to encode a phosphoinositide 3-kinase that specifically phosphorylates phosphatidylinositol to produce phosphatidylinositol 3-monophosphate (PI(3)P) [61]. Temperature-sensitive *vps34* mutant cells exhibit an immediate defect in protein sorting to the vacuole strongly supporting a role for the Vps34 PI 3-kinase in vesicular transport from the Golgi to the vacuole [61].

How does PI(3)P regulate vesicle-mediated protein transport? Recent work has identified a set of effector molecules that bind directly to this lipid. One of these, the human early endosomal antigen (EEA1) protein, is a peripheral membrane protein that interacts with the active form of the Rab5 GTPase [62]. EEA1 must associate with membranes in order to stimulate homo- and heterotypic early endosome fusion — an interaction which requires the presence on the membrane of PI(3)P, active Rab5, and

the FYVE domain of EEA1. Several groups have now shown that for EEA1 and other FYVE domain containing proteins such as Hrs-1, Vps27p, Vac1p, Fab1p, and Pib1p, the FYVE domain can directly and selectively bind PI(3)P. This interaction has been shown to target these proteins to endosomes *in vivo* [36,63,64] (Figure 2). Thus, the FYVE domain represents a conserved adaptor domain whose primary function is to act as a membrane-attachment module that depends upon interaction with PI(3)P. High affinity binding of FYVE domains to PI(3)P seems to depend on the intact lipid and its presence within a membrane structure. This is in contrast to phosphoinositide-binding to the PH domains from PLC-δ1, Grp1 and Btk, which are almost exclusively driven through interactions with the inositol phosphate head group.

Following nuclear magnetic resonance (NMR) studies of the EEA1 FYVE domain in complex with di-C_4-PI(3)P, as well as observations of its interaction with PI(3)P-containing mixed micelles, Kutateladze and Overduin [65] have suggested a three-step mechanism for the interaction of the FYVE domain with the PI(3)P-containing membrane. An exposed hydrophobic loop formed from residues immediately preceding the FYVE signature motif — a region well conserved among FYVE domains — initially contacts the membrane in a non-specific manner. This causes a small conformational change opening up the basic PI(3)P-binding pocket, which then binds to the head group of PI(3)P [65]. This study argues that polar interactions with the PI(3)P head group and hydrophobic interactions with the membrane interior cooperate to drive high-affinity binding of the FYVE domain to PI(3)P-containing membranes. Thus, the ability of FYVE domains to penetrate the membrane surface is likely to explain the difference between FYVE and PH domains in the extent to which the inositol phosphate head group drives membrane binding.

Given the specificity of the FYVE domain for PI(3)P [63,64] a number of groups have tried to use this domain as a probe to visualise the intracellular localisation of PI(3)P. However, in the absence of the adjacent Rab5 binding domain, the EEA1 FYVE domain is cytosolic, indicating that it binds with too low an affinity for PI(3)P to be a useful probe [66,67]. This problem has been overcome by the use of a tandem FYVE domain of the hepatocyte growth factor-regulated tyrosine kinase substrate, Hrs. When expressed in mammalian cells, this probe revealed that PI(3)P is undetectable in most intracellular membranes of fibroblasts, including the Golgi and the plasma membrane [68]. However, PI(3)P is abundant on the limiting membranes of early endosomes, and on the membranes of intralumenal vesicles of multivesicular endosomes [68].

Over 30 different proteins have now been identified that contain a FYVE domain [63]. Rabenosyn-5 is a newly identified FYVE domain containing protein which, like EEA1, is required for homo- and heterotypic early endosome fusion and binds Rab5-GTP and PI(3)P [69]. Another recently identified FYVE domain protein, Rabip4, binds specifically to Rab4–GTP and is localised to the early endosomes [70]. Whereas Rabenosyn-5 appears to regulate transport of lysosomal enzymes from the *trans* Golgi network (TGN) to the endocytic pathway, Rabip4 appears to be involved in the control of recycling from early endosomes [70]. Thus, at least with these cases and with EEA1, the FYVE domain binding to PI(3)P coupled with the all important interaction with distinct Rab proteins can induce a certain degree of directionality in endocytic membrane traffic.

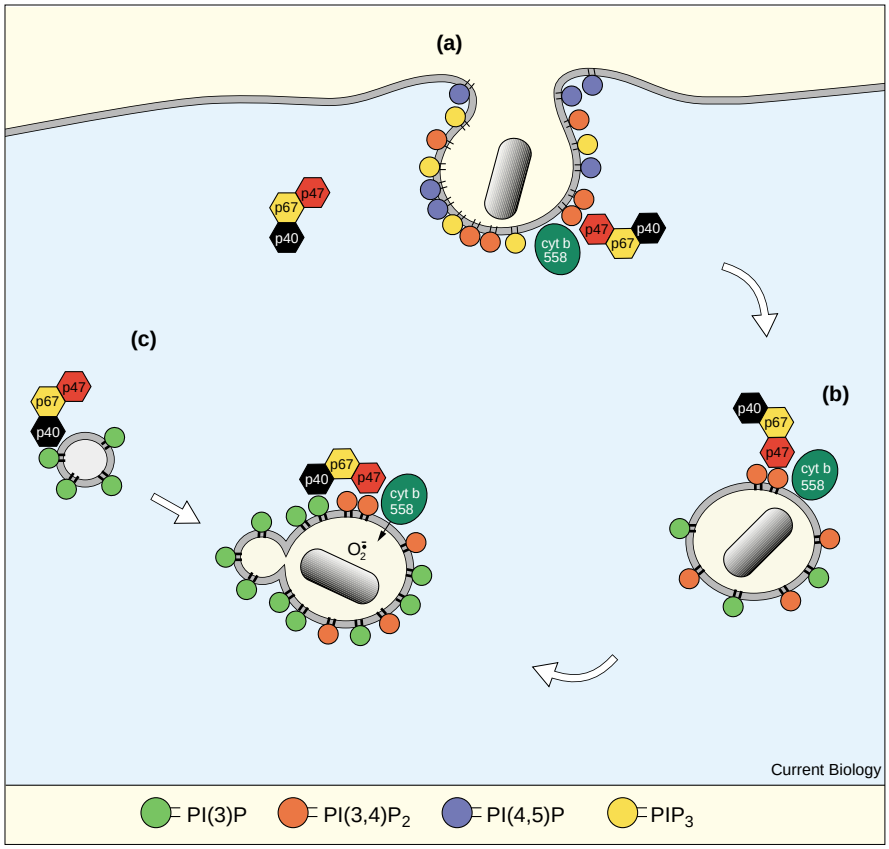
Other FYVE domain-containing proteins include enzymes that metabolise PI(3)P such as the phosphatidylinositol 3-phosphatase FYVE-dual specificity phosphatase 1 (FYVE-DSP1) [71] and the mammalian orthologue of the yeast PI(3)P 5-kinase Fab1, PIKfyve [72]. This kinase phosphorylates PI(3)P into phosphatidylinositol 3,5-bisphosphate (PI(3,5)P_2) and has been localised to multivesicular bodies indicating a role for PI(3,5)P_2 in the late endocytic pathway. This is in line with previous data showing that mutations in Fab1 result in an expanded vacuole [73]. At present however, our understanding of this lipid is hampered by the lack of identified PI(3,5)P_2 effectors. One approach to addressing this issue may be through examining whether specific PI(3,5)P_2-binding PH, FYVE or PX domains (see below) can be identified. Irrespective of this, it is clear that regulation of the levels of 3-phosphoinositides is vitally important for proper endocytic membrane trafficking.

PX domains: the new kids on the block

Such a conclusion has been reinforced following the recent demonstration that PX domains function as phosphoinositide-directed membrane targeting motifs [74–79], (reviewed in [80,81]; Figure 2). The PX domain was initially identified as a conserved motif of approximately 130 residues within the $p40^{phox}$ and $p47^{phox}$ subunits of the neutrophil NADPH oxidase superoxide-generating complex [82]. PX domains can be found in a wide variety of proteins involved in cell signalling (phospholipase D1 and D2, class II PI 3-kinases), vesicle trafficking (human sorting nexins, yeast Vam7p), and control of yeast bud emergence and polarity (Bem1p and Bem3p). Currently, at least 57 human and 15 yeast proteins that contain PX domains have been identified [80,81].

Vam7p is an essential component of vacuolar sorting in yeast helping to transport cargo such as hydrolytic enzymes to the vacuole [83]. Vam7p is a soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (t-SNARE) that contains an amino-terminal PX

Figure 5



Phosphoinositide turnover during phagocytosis. **(a)** Interaction of the particle with cell surface receptors causes recruitment and activation of PI 3-kinaseγ and a rapid local production of PIP_3 on the phagocytic cup. The action of the SHIP phosphatase leads to a concomitant local increase in PI(3,4)P_2. Type II PI(4)P 5-kinase is also recruited, causing PI(4,5)P_2 levels to rise. **(b)** On closure of the phagocytic cup, the PIP_3 levels fall rapidly, as does the level of PI(4,5)P_2. **(c)** PI(3)P-containing early endosomes fuse with the vacuole causing a further elevation of PI(3)P. The NADPH oxidase complex is now bound to PI(3)P through the PX domain of its $p40^{phox}$ subunit and to PI(3,4)P_2 through the PX domain of its $p47^{phox}$ subunit. These subunits interact with the constitutively membrane-associated cytochrome b_{558} forming a fully active complex that directs toxic superoxide production into the phagocytic vacuole.

domain. It is targeted to vacuoles, where it acts as a SNAP-25 homolog in the docking and fusion of transport intermediates with the vacuole [84]. Recently, it has been shown that the Vam7p membrane association requires a functional PX domain and PI(3)P generation *in vivo*, and that the Vam7p PX domain binds to PI(3)P *in vitro* [74]. Thus the PX domain is likely to concentrate Vam7p at its site of action through an inherent ability to bind PI(3)P.

Further evidence for the role of the PX domain as a PI(3)P directed membrane-targeting module has been provided by the analysis of the human sorting nexin, SNX3 [77]. Sorting nexins are an emerging family of widely expressed proteins believed to be part of the complex molecular machinery required for protein trafficking — currently more than 16 SNXs have been identified [84–87]. Although the precise functions of these proteins are unclear, sorting nexins appear to target cargo proteins such as growth factor receptors to specific organelles for post-translational modification, degradation or recycling. Thus, overexpression of SNX1 — initially identified through its ability to bind the cytosolic domain of the EGF receptor [88] — selectively decreases the amount of EGF receptor on the cell surface by enhancing the rate of constitutive and ligand-induced EGF receptor degradation.

As with Vam7p, the PX domain of SNX3 specifically binds PI(3)P, an interaction that targets this protein to early and recycling endosomal compartments [77]. In A431 cells, overexpression of SNX3 shifts the steady-state distribution of transferrin receptor (TfR) from the surface to a SNX3-positive tubular-vesicular structure. More detailed analysis has revealed that proteins of the early endosome (EEA1), the recycling endosome (TfR), and the late endosome (mannose-6-phosphate receptor and syntaxin) are all present in this structure. This protein distribution indicates that SNX3 overexpression induces a mixing of the early, recycling and late endosomal compartments. Overexpression of SNX3 mutants defective in PI(3)P-binding, showed they were cytosolic and the surface level of TfR remained unaltered, strongly suggesting that the PI(3)P-binding activity of SNX3 is necessary for its endosomal association, which in turn enables SNX3 to regulate the structure and function of the endosomal pathway. Further work will be required to elucidate the molecular basis for the SNX3 induced expansion of tubular-vesicular structures.

The identification of the PX domain as a phosphoinositide-binding module provides significant insight into the function of this domain in targeting its host protein to specific cellular membranes. However, ascribing such a function to the PX domain raises a number of interesting questions about specificity and regulation. For example, as the PX motif is found in a wide variety of proteins, it is interesting to consider whether individual PX domains are capable of specifically recognising phosphoinositides other than PI(3)P — an important issue which will likely affect the subcellular targeting of the host protein (see below). In *Saccharomyces cerevisiae* this does not appear to be the case [89].

PX domains and phagocytosis

Phagocytic cells such as neutrophils and macrophages engulf and destroy invading microorganisms through a receptor-mediated mechanism that involves extensive cytoskeletal rearrangements and membrane remodelling [90]. Receptors on the phagocyte surface that recognise surrounding components of the microorganism, or opsonins attached to it, cluster in the area of contact between the pathogen and phagocyte. Such clustering triggers localised actin assembly and the formation of a phagosomal cup [90] (see Figure 5). Following closure of the cup, activation of the multi-subunit phagocyte oxidase (Phox) complex on the phagosomal membrane kills the invading microorganism with a cocktail of reactive oxygen species [90]. As mentioned above, prominent among the signals regulating phagocytosis are rapid changes in phosphoinositide metabolism [12,91].

There is strong evidence that localised accumulation of PIP_3 around the phagocytic cup, through activation of class I PI 3-kinases [91], regulates phagosome closure, possibly through small GTPases of the Rho and ARF family [90]. More recently, Ellson and co-workers [92,93] have shown that when a macrophage cell line expressing a GFP probe specific for PI(3)P is challenged with IgG-opsonised zymosan particles, there is a dramatic accumulation of the probe on the phagosomal membrane following phagosomal closure, consistent with PI(3)P being rapidly synthesised on phagosomal membranes. Such a dramatic accumulation of PI(3)P around the entire phagosome suggests that PI(3)P is clearly an ideal candidate for controlling the early stages of phagosomal maturation.

How such a rise in PI(3)P is achieved is currently unclear. One possibility is that PI(3)P is produced by the sequential breakdown of PIP_3 and phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$) by a 5- and 4-phosphatase, respectively. This is consistent with the production of PIP_3 prior to phagosomal closure [91] and the recruitment of the PIP_3 5-phosphatase (SHIP) to phagosomal membranes [94]. The other two possible routes for PI(3)P production are through direct phosphorylation of phosphatidylinositol by either class II or class III PI 3-kinases on the phagosomal membrane. In this context it is interesting that following phagosome closure, Ellson *et al.* [92] observed the docking and fusion of early endosomes with the nascent phagosome. This occurred prior to the large global increases in phagosomal PI(3)P levels and may represent a mechanism for delivery of an active PI 3-kinase to the phagosomal membrane. Furthermore, Vieira *et al.* [95] have now confirmed the presence of class I PI 3-kinase activity in the nascent phagosomal cup and have shown that the class III PI 3-kinase Vps34 is responsible for PI(3)P synthesis on phagosomal membranes. The latter process is essential for phagosome maturation.

What is the relevance of such dynamic changes in PI(3)P for phagocytic function? Recent studies [75,76] have shown that two components of the phagocytic oxidase complex, $p40^{phox}$ and $p47^{phox}$, contain PX domains that specifically bind PI(3)P and $PI(3,4)P_2$, respectively. This raises the possibility that the elevation in PI(3)P — and perhaps $PI(3,4)P_2$ — may be the signal that recruits these proteins to the phagosome. The PI(3)P driven assembly of $p47^{phox}$, $p40^{phox}$ and the associated $p67^{phox}$ with the constitutively membrane-bound components of the oxidase complex would result in a functional oxidase capable of producing the toxic oxygen metabolites needed to kill the invading microorganisms. The importance of this interaction is highlighted by the observation that one of the mutations associated with patients suffering from chronic granulomatous disease (these patients have a strong susceptibility to bacterial and fungal infections) is found in the PX domain of $p47^{phox}$; this mutation abolishes its binding to $PI(3,4)P_2$ [75].

Intriguingly, PI(3)P has also been seen to accumulate on the membranes of another specialised endocytic structure. When *Salmonella* invade cells they become enclosed in a membrane-bound compartment termed a *Salmonella* containing vacuole. This compartment appears to share some features with early endosomes, but provides a privileged environment in which the bacteria can divide rather than be delivered to late endosomes and lysosomes [96]. The use of another FYVE domain GFP probe has shown that PI(3)P accumulates in both the cell surface ruffles that emerge upon *Salmonella* binding and in the membranes of the vacuoles [97]. Given that there is a rapid accumulation of PI(3)P on phagosome membranes, this accumulation on *Salmonella* containing vacuole membranes may reflect a cellular response to the invading bacteria.

It is of note that the accumulation of PI(3)P on *Salmonella* containing vacuoles occurs even in the presence of the PI 3-kinase inhibitor wortmannin, showing that a wortmannin resistant PI 3-kinase is involved in the process [97]. Furthermore, binding of the FYVE–GFP probe to *Salmonella*

containing vacuoles was periodic: the vacuoles appeared to 'flash' as they gained and lost the GFP probe. These data suggest that the levels of PI(3)P on the vacuole membranes fluctuate. It is possible that this represents a 'battle' between the invading bacteria manipulating the composition of the vacuole to lower PI(3)P levels and the host cell trying to maintain high levels of PI(3)P on the membranes and thus target the bacterium for destruction. Destruction occurs either by recruitment of the phagocytic oxidase system or delivery of the vacuole to later points along the endocytic pathway.

The above data show that just as pathogenic mycobacteria appear to be able to arrest phagosome maturation by reducing available PI(3)P in the phagosome membrane [98], *Salmonella* apparently do a similar thing in bacterium-containing vacuole membranes. The fact that, as a means of evading host cell defences, both of these pathogens target PI(3)P levels in the host cell membranes that envelop them underlines the importance of this phosphoinositide, and the proteins that interact with it, in the pathways from the cell surface to various endosome destinations.

Conclusions

An emerging and in some cases well developed theme in phosphoinositide signalling is their role as dynamic scaffolds for the regulated formation of membrane associated signalling complexes. The more historical concept of 'rigid' signalling envisages receptors and other signalling proteins being quite fixed, and spatial signal transmission proceeding *via* the rapid diffusion of second messengers. In contrast, signalling through phosphoinositides is a highly dynamic process that is principally reliant upon networks of low affinity interactions; it is therefore amenable to both rapid and subtle manipulation. A key aspect of the signal transmission process is signalling proteins that undergo substantial translocation and reversible binding interactions. These signalling proteins move to and act at defined locations specified by the local phosphoinositide environment — arguably a more 'soft' form of signalling.

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