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Polyphosphoinositide binding domains: key to inositol lipid biology

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Abstract

Polyphosphoinositides (PPI_n) are an important family of phospholipids located on the cytoplasmic leaflet of eukaryotic cell membranes. Collectively, they are critical for the regulation many aspects of membrane homeostasis and signaling, with notable relevance to human physiology and disease. This regulation is achieved through the selective interaction of these lipids with hundreds of cellular proteins, and thus the capability to study these localized interactions is crucial to understanding their functions. In this review, we discuss current knowledge of the principle types of PPI_n-protein interactions, focusing on specific lipid-binding domains. We then discuss how these domains have been re-tasked by biologists as molecular probes for these lipids in living cells. Finally, we describe how the knowledge gained with these probes, when combined with other techniques, has led to the current view of the lipids' localization and function in eukaryotes, focusing mainly on animal cells.

Keywords

Phosphoinositides; PH-domain; PX-domain; phospholipase C; fluorescence imaging; membrane

Inositol containing phospholipids have played a fundamental role in eukaryotic evolution (see the review by Michell in this issue). Most of their biology focuses on phosphorylation of the inositol head group in phosphatidylinositol (PtdIns), which yields a family of seven polyphosphoinositides (PPI_n; we use the nomenclature proposed by Michell [1]; see figure 1). These PPI_n regulate a truly impressive number of eukaryotic functions – as can be gleaned from the other articles present in this volume, and as recently comprehensively reviewed [2]. How can a family of chemically simple phospholipids control such an impressive array of biological functions? The answer in all cases lies in their ability to interact with and thereby regulate proteins. Details of specific protein interactions and their functions are best left in their biological context as described in detail in the accompanying reviews. Rather, our goal in this review are threefold: (i) to outline the general principles of PPI_n-protein interactions, (ii) to discuss how lipid binding proteins can be used as biosensors to study PPI_n localization and dynamics in living cells and finally (iii) to provide

an overview on the current state-of-the-art with regards our knowledge of the lipids' cellular distribution.

General Principles of PPIIn-protein interactions

A break-through in understanding how PPIIn execute their biological functions came with the discovery of high affinity PtdIns(4,5) P_2 binding by the isolated Pleckstrin Homology (PH) domain from phospholipase C delta [3,4]. This example was rapidly followed by the discovery of other PH domains with high affinity but diverse PPIIn-binding selectivities [5], as well as by the identification of other PPIIn-binding domains such as the FERM [6,7], FYVE [8–10], PX [11–13], ENTH/ANTH [14,15], PROPPIN [16] and the recently described TRAF [17] domains. These observations together revealed the first and canonical principle of PPIIn-protein interactions (Figure 2): that high-affinity PPIIn binding by conserved families of lipid binding domains drives membrane recruitment of proteins, thereby regulating protein activity in time and space [5].

This first principle remains fundamentally important for understanding PPIIn function. Indeed, high-affinity binding of PtdIns3 P appears to be a shared function of every PX domain found in *Saccharomyces cerevisiae* [18]. However, this general principle is not universal; for example, the PH domain family counts relatively few members that exhibit selective and high affinity PPIIn binding [19]. Instead, most members of this large family are now thought to be more general scaffold modules, regulating diverse inter-molecular interactions [20]. Although many PH domains exhibit some binding to PPIIn, more often than not the affinity is too low to dictate protein localization [19]. Does this make PPIIn binding irrelevant? Probably not, as illustrated by the PH domains found in the cytohesin family of ARF guanine nucleotide exchange factors. Cytohesins come in two splice variants: a “2G” variant that exhibits high-affinity binding to PtdIns(3,4,5) P_3 that can direct protein localization [21–23]; and a second, “3G” splice variant with an additional glycine residue that expands the lipid binding pocket in the PH domain, reducing both specificity and affinity [21,24] to such an extent that PPIIn binding cannot drive membrane localization [25]. Instead, additional protein-protein interactions between the cytohesin PH domains and Arf/Arl family GTPases have been discovered, which cooperate with PPIIn binding to target the cytohesins to membranes [26–28].

This illustrates a second principle of PPIIn-protein interactions, one that is an extension of the first: PPIIn binding by specific domains can add the necessary avidity to a second interaction that together drive membrane binding (Figure 2). This principle has also been coined “co-incidence detection” [29], and is widely used. For example, interaction between PX domains and PPIIn combined with membrane curvature sensing by BAR domains is a central mechanisms by which sorting nexins separate and route different cargos in the endocytic pathway [30]. This combinatorial approach whereby multiple protein or lipid ligands as well as physical constraints such as membrane curvature can direct protein function is a powerful and adaptable one, but can also generate significant caveats when trying to interpret the role of PPIIn binding in protein localization, as we will see in the next section.

Note that recruitment of cellular proteins to cell membranes through PPIIn binding is not a unique feature of eukaryotic cells; many bacterial pathogens contain protein binding domains that target their host cell's membrane compartments using PPIIn interactions (see the review by Cossart in this issue). These include a family of PtdIns4*P*-binding proteins in *Legionella pneumophila* [31,32] and a unique and widely distributed bacterial PPIIn binding domain (BPD) found in many type III secretion system-containing bacterial pathogens [33].

There is also a third general principle whereby PPIIn contribute to membrane targeting, albeit in a less specific manner. Many proteins contain polybasic domains, which when coupled to some hydrophobic character (for example, bulky hydrophobic residues or lipid modifications), selectively partition onto the plasma membrane [34]. Membrane binding depends on electrostatic interactions with anionic lipids that are enriched on the inner leaflet of the plasma membrane [34,35], principally phosphatidylserine (PtdSer) [35,36]. However, PtdSer enrichment at the plasma membrane alone is not sufficient for localization of polybasic domains, which require the presence of PPIIn, mainly PtdIns(4,5)*P*₂ and PtdIns4*P* [37,38]. This observation is reflected by the capacity of polybasic domains to sequester PtdIns(4,5)*P*₂ molecules in the plane of a PtdSer-rich membrane *in vitro* [39]. Therefore, it appears that PPIIn satisfy a polyanionic lipid requirement for polybasic domain binding in the unique electrostatic environment of the plasma membrane. Interestingly, in addition to unstructured polybasic domain containing proteins, the Kinase-Associated-1 (KA-1) domain family has recently been demonstrated to exploit a similar electrostatic lipid binding mechanism to specifically target the plasma membrane [38,40].

Aside from simple membrane binding, PPIIn can also assist in direct conformational activation of proteins. Association of PPIIn ligands with the PH domains from PKB [41], Arf GAPs [42,43] and the Arf GEF Brag2 [44] activate the proteins independently from effects on membrane targeting. Conformational activation is also the mechanistic principle by which many integral membrane channels and transporters are regulated by PPIIn [45]. Indeed, recent structural studies have revealed how PtdIns(4,5)*P*₂-binding at the interface between the transmembrane and cytosolic domains contributes to opening of the cytosolic pores of two types of voltage gated inwardly rectifying potassium channels [46,47].

Finally, recent work has produced compelling evidence for binding of both the hydrophilic head along with the acyl tails of PPIIn to lipid transfer domains, facilitating non-vesicular transfer of these molecules between membranes [48–50]. If coupled to PPIIn phosphorylation and de-phosphorylation reactions nestled on separate membranes between which the lipid species is transferred, a chemical gradient may be established; this has been proposed to provide the energy for an antiporter mechanism that drives non-vesicular transport of other lipids [50]. Whilst this exciting idea has not yet been firmly established, with alternative scenarios involving lipid sensing rather than transfer [51], it seems at least possible that this mechanism represents a newly discovered principle by which PPIIn control cellular activity [52].

In summary, at present we can identify five functional principles by which PPIIn regulate their binding proteins (see Figure 2): (i) direct recruitment of a protein to a membrane by high affinity stoichiometric binding. (ii) “Co-incidence detection” whereby PPIIn cooperate

with other molecular interactions to specify the membrane location where recruitment occurs. (iii) Simple electrostatic interaction with a protein in the context of a general anionic lipid environment. (iv) Allosteric activation induced by binding and finally (v) through interactions with lipid transfer proteins to facilitate non-vesicular lipid transport. Through these five functional principles, the PPIIn can control protein activity in space (by directing protein activity on specific membranes) and time (through modulation of PPIIn levels in these membranes). It follows that to fully appreciate the regulation of cellular physiology by these lipids requires an understanding of their dynamic distribution in cells. We therefore next turn to how PPIIn-protein interactions can be utilized as experimental tools to address this precise issue.

PPIIn binding proteins as biosensors - PPIIn localization

Specific, high-affinity binding of PPIIn by isolated domains with 1:1 stoichiometry is a molecular talent poised for exploitation, since these domains can be cloned, engineered and re-tasked as biosensors for detecting PPIIn. Purified recombinant proteins have been used successfully by many groups to probe for PPIIn in fixed cells by both fluorescence and electron microscopy. These have included probes against PtdIns(4,5) P_2 [53–56], PtdIns(3,4) P_2 [57], PtdIns(3,4,5) P_3 [58], PtdIns5 P [59] and PtdIns3 P [60]. Such approaches often provide quantitative data, especially when validated against biochemical measurements of lipid amounts [38,53,57,58,61] or appropriate standard curves [54]. However, the preservation of lipids and membranes for fluorescence or electron microscopy is a tricky business (see the respective discussions of each in [55] and [53]). Thus a considerable amount of empirical determination is required to ensure the detected signals represent a pool of PPIIn present in its native compartment, which must remain reasonably intact despite fixation and the rendering internal membranes accessible to externally applied probes via permeabilization or mechanical disruption. These requirements obviously preclude interrogation of the dynamic distribution of the lipids in living cells.

An alternative-and indeed, pioneering approach [62,63]-to using PPIIn binding domains as biosensors was through their fusion to fluorescent proteins; these can be ectopically expressed in cells or even whole organisms, typically exhibiting a diffuse cytosolic/nuclear distribution, but with marked enrichment on specific membrane compartment(s) (e.g. Figure 3). For a high quality biosensor, the membrane enrichment will depend on the presence of a specific PPIIn species *and only on that species*. The sensor will thus report on dynamic changes of the PPIIn concentration in this and any other compartment, to an extent that depends on the domain's binding affinity. Thus fluorescent lipid biosensors have the capacity to reveal the dynamic distribution of PPIIn pools in living cells in real time at the resolution limit dictated by the microscope. The downside, as recognized early on in the use of these probes [64], is that rarely if ever are all the criteria met for a specific and unbiased probe; it is always important to be aware of confounds that impact PPIIn biosensor behavior, as we will discuss below.

Table 1 lists the seven PPIIn species, some of the commonly used probes to image them and the cellular localization revealed by each probe. We also list three crucial criteria that we believe should be taken into account when interpreting data from each probe: Is the

biosensor specific for the target PPIIn? Is its localization in cells dependent on this PPIIn? And if is dependent on that PPIIn, is the lipid alone sufficient to drive localization? As can be seen from even a cursory glance of the table, very few biosensors satisfy all three of these criteria. However, all have their merit and have yielded useful data even when their individual caveats are taken into account.

The first criteria (binding specificity) has typically been the gold standard when assessing the suitability of probes, and is usually determined by *in vitro* experiments. These *in vitro* assays have their own host of caveats that are beyond the scope of this review (see an excellent discussion in [65]); in short, different assays can yield different results, and are rarely definitive in isolation. A battery of different assays yielding congruent data is usually required to gain confidence that the true specificity has been determined. Even so, many biosensors that fail under this criteria have still been extremely useful and selective probes. For example, the PtdIns(4,5) P_2 biosensor derived from the Tubby c-terminal domain shows no selectivity for this PPIIn over PtdIns(3,4) P_2 and PtdIns(3,4,5) P_2 [66]. However, the 20 fold excess of PtdIns(4,5) P_2 over these other lipids in cells [67] means that PtdIns(4,5) P_2 is the only lipid at sufficient concentration to drive localization of the probe (Table 1). Likewise, a biosensor based on three tandem repeats of the Ing2 PHD domain recognizes both PtdIns3P and PtdIns5P *in vitro*. However, the probe does not detect cellular PtdIns3P pools, and can detect PtdIns5P synthesis in the plasma membrane [68]. Unfortunately, the affinity of this biosensor seems insufficient to detect the scant endogenous PtdIns5P levels.

A frequent impediment to specificity is binding of PH domains to the equivalent soluble inositol phosphate as forms the hydrophilic “head” of the phospholipid. This is only a concern when these inositol phosphates accumulate at sufficient concentration to compete with the lipid (which can be a serious issue in the case of Ins(1,4,5) P_3 , as we will discuss later). Furthermore, additional membrane binding interactions often favor lipid binding over soluble inositol phosphates in the physiological environment [69,70].

In our view, these examples demonstrate the equal importance of the second two criteria that address behavior in cells. The dependence on PPIIn binding is readily assessed in most cases by inducing acute depletion of the lipid and determining whether the probe’s localization is lost; sufficiency can then be assessed by ectopically driving lipid synthesis and determining whether the probe detects this ectopic synthesis. For example, Figure 3 shows that depletion of PtdIns(4,5) P_2 from the plasma membrane by chemically-induced recruitment of a 5-phosphatase (see the detailed discussion on these inducible tools by De Camilli and Idevall-Hagren) causes depletion of the biosensors PH-Plcd4, demonstrating dependence on this lipid for membrane localization in live cells. Although a crucial control, this doesn’t exclude the possibility that another factor assists in membrane targeting (i.e. it is a “co-incidence detector”), biasing the probes towards reporting on PtdIns(4,5) P_2 only at the plasma membrane. However, Figure 3 also reveals that induction of ectopic PtdIns(4,5) P_2 synthesis at late endosomes is sufficient to the probe, arguing strongly that PtdIns(4,5) P_2 alone is sufficient to target the biosensor.

The importance of considering whether a particular PPIIn is sufficient as well as necessary to explain localization of a PPIIn biosensor is well illustrated by the large family of PtdIns4P

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biosensors (Table 1). The first two biosensors, those derived from the PH domains of OSBP and FAPP1, display no particular preference for PtdIns4P over PtdIns(4,5)P₂ in most, but not all, *in vitro* assays [71–74]. Nevertheless, when expressed as fluorescent protein fusions in cells both probes label Golgi membranes in a PtdIns4P-dependent fashion; maneuvers that cause selective depletion of PtdIns4P from this compartment release the biosensors from the membrane [71,75,76]. It is easy to interpret these data as indicative that the probes are not influenced by PtdIns(4,5)P₂ binding in cells, and that the Golgi localization reflects the unique compartmental accumulation of PtdIns4P. However, it is clear that these probes utilize the co-incidence detection mechanism, and targeting also depends on the presence of Arf1 [71,75,77]. Therefore, the distinct Golgi localization reflects a unique accumulation of both molecular species (Arf1 and PtdIns4P) at these membranes. Indeed, albeit with a less pronounced accumulation, the OSBP PH domain also labels the plasma membrane in a PtdIns4P-dependent and PtdIns(4,5)P₂-independent manner [75,78].

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An attempt to circumvent this biased detection of Golgi-localized PtdIns4P pools led Roy et al to examine binding sites for potential non-lipid ligands in PH domains, allowing them to identify the *S. cerevisiae* Osh2p PH domain as a PtdIns4P binder that did not depend on an additional protein ligand for membrane targeting [72]. Although like its OSBP and FAPP1-derived predecessors, this domain exhibits PtdIns(4,5)P₂ binding *in vitro*, it was shown to depend only on PtdIns4P to bind to membranes in yeast cells, and crucially to detect both plasma membrane and Golgi pools of this lipid [72]. When expressed in mammalian cells as a tandem dimer of two identical PH domains (to increase avidity), this probe selectively labels the plasma membrane, co-dependent on the presence of PtdIns4P and PtdIns(4,5)P₂ [38,72,79]. Its failure to detect PtdIns4P in the Golgi, combined with the capacity of PtdIns(4,5)P₂ alone to suffice for plasma membrane localization [38] led us to conclude that it fails under all three criteria as a PtdIns4P biosensor (Table 1). On the other hand, we stress that the probe works well in yeast (perhaps owing to this organism's lower relative PtdIns(4,5)P₂ accumulation), and the conclusion that the large PtdIns4P accumulation in *sac1* null cells is at the plasma membrane is certainly valid [80].

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Recently, we have investigated the capacity of the high-affinity PtdIns4P binding domain from SidM (P4M) for its capacity as a biosensor for this lipid. P4M exhibits excellent specificity and affinity for PtdIns4P in a variety of *in vitro* assays [32,81], satisfying our first criterion (Table 1). The probe labels several compartments including the plasma membrane, Golgi and endo-lysosomal network [82], which in all three cases release the probe after acute depletion of PtdIns4P with a Sac phosphatase (second criteria). Crucially, however, induction of ectopic PtdIns4P synthesis on ER-derived membrane via a Hepatitis C virus non-structural protein 5A/PI4KA complex is sufficient to recruit the probe, satisfying our third criteria (Table 1). This gave us the confidence to conclude that P4M reveals the major steady-state pools of PtdIns4P in a typical mammalian cell [82], a feat not achieved by any of the contemporary biosensors.

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Efforts to create a PtdIns(3,5)P₂ biosensor have been frustrated. The high-affinity PROPPIN binding domain of Atg18p (erstwhile known as Svp1p) has exquisite specificity and affinity *in vitro*, but is almost certainly biased by additional molecular interactions *in vivo* [16]. A recent report proposed use of the cytosolic N-terminal domain from the mucolipin transient

receptor potential channel (TRPML1) as a biosensor; the probe was reported to show good specificity for PI(3,5)P₂ *in vitro*, and to exhibit dependence on this lipid for localization in cells, based on reduced co-localization with late endo-lysosomal markers after pharmacological or genetic depletion of the lipid [83]. However, our own efforts using time-lapse imaging in live cells show no release of the probe after PI(3,5)P₂ depletion with pharmacological or enzymatic means (G.R.V.H. and T.B., unpublished observations), and for this reason we list this biosensor as ambiguous with respect to satisfying the cellular criteria in Table 1.

In summary, we encourage a critical approach when interpreting the localization of PPIIn biosensors, and it has been rare to identify probes which generate definitive answers to a particular lipids' cellular distribution. We have attempted to fill some of the gaps in the criteria we propose to assess fidelity of these probes in Figure 3. Although there is certainly room for the development of new and improved biosensors, there are high-quality probes available for most of the PPIIn (as listed in Table 1).

PPIIn binding proteins as biosensors-Quantitation

So far, we have discussed the capacity of biosensors to localize particular pools of PPIIn. Yet this is only half the story; the capacity of cells to dynamically alter the concentration of a PPIIn is crucial to temporal modulation of protein function, and is the central mechanism underlying these lipids' signaling functions. Prior to the advent of biosensors, biochemical measurements based on isotopic labeling or chemical determination of mass were required for quantitation of PPIIn from cells [84], which have recently been joined by advances in lipid mass spectrometry [85]. Although these methods are accurate, they are unable to provide single cell resolution, and sub-cellular resolution requires laborious and artifact-prone fractionation. The newly emerging methodology of imaging mass spectrometry using magnetic sector secondary ion mass spectrometry offers great promise for solving these problems in the near future [86]. In the meantime, fluorescent biosensors provide a readily available solution, with the added advantage that PPIIn dynamics can be monitored in living cells. For an ideal biosensor meeting each criteria from Table 1, the extent of membrane localization will vary depending on the concentration of PPIIn. How quantitative is this relationship?

Before we can answer this question, we need to consider the potential for these biosensors to fail as passive and unobtrusive reporters for their target lipids. Most of the biosensors listed in table 1 are single domains that bind the lipid with a 1:1 stoichiometry and a characteristic dissociation constant, K_d . Some are tandem dimers or even trimers of such domains, increasing their avidity with the consequent multiplicative enhancement of the effective K_d . In either case, for the sensors to be able to detect a cellular pool of PPIIn then the unbound, free lipid must be present at concentrations $\sim K_d$ -averaged across the entire volume of the cytoplasm for 1:1 binding, or measured locally in the plane of the membrane for probes with multiple lipid binding sites. Problems can arise as the concentration of biosensor expressed in the cell approaches or even exceeds the K_d , since in this case a substantial fraction of the free PPIIn will be sequestered by the biosensor. This can lead to competition with and ultimately displacement of endogenous PPIIn binding proteins, and result in disruption of

their physiological function. At present, we have very little information on the homeostatic mechanisms that regulate the steady state accumulation of PPIIn-especially the more abundant PtdIns(4,5) P_2 , PtdIns4 P and PtdIns3 P lipids. It is possible that cells can sense the free lipid concentration and adjust synthesis to maintain this level if a fraction becomes sequestered by an over-expressed binding protein-but to our knowledge this has never been definitively demonstrated. What has been shown is that over-expression of PtdIns(4,5) P_2 biosensors can lead to inhibitory effects on PLC signaling [63,87] and exocytosis [88]. Indeed, inhibition can be a useful test of the role of a PPIIn in a cellular process. This consideration is especially important for PPIIn that accumulate in low abundance such as PtdIns(3,4,5) P_3 , since biosensors exhibit sub micromolar K_d [89,90] and are known to produce inhibitory effects [91].

Regardless of whether a significant fraction of the free PPIIn is bound, it is important to consider what a lipid biosensor actually reports on: a fraction of the endogenous PPIIn, sampled from the free PPIIn pool but that is now in complex with the biosensor. This has two fundamental consequences: Firstly, PPIIn molecules that associate with endogenous effector proteins, which as we discussed in the first section execute all of the lipids' biological functions, are invisible to the biosensor. Consider the interaction of the clathrin mediated endocytosis machinery with PtdIns(4,5) P_2 in the plasma membrane (See the review by Haucke in this issue); as these proteins oligomerize they may sequester and concentrate PtdIns(4,5) P_2 . Because this local PtdIns(4,5) P_2 is sequestered by a high local concentration of binding proteins, the free PtdIns(4,5) P_2 concentration could be much lower than outside the clathrin-coated pit and therefore – perversely – the biosensor could report a *lower* local enrichment of lipid. Secondly, as soon as a biosensor binds a lipid, any other interactions with the inositol ring are occluded; since these biosensor: lipid complexes exhibit rapid diffusion $\sim \mu\text{m/s}$ with substantial binding times ($\sim\text{s}$) [92–94], they have the potential to “smear out” local heterogeneities in local PtdIns(4,5) P_2 over a micron scale. This is particularly important to bear in mind when attempting to utilize these tools with methods that localize the lipid with high resolution (i.e. both electron and “super-resolution” light microscopy).

The concentration of biosensor present is rarely determined, but may typically reach micromolar levels [95]. Therefore, the minimum expression levels required to produce acceptable signal to noise in images is strongly advised. This criteria becomes increasingly less difficult to satisfy with ongoing advances in camera and photomultiplier tube sensitivity. For the time being, we will assume that the concentration of biosensor is much less than the effective PPIIn concentration. In this case, we can think of the lipids as ligands in excess binding to biosensor “receptors”; the fraction of biosensor bound can then be estimated as $[\text{PPIIn}]/K_d + [\text{PPIIn}]$, where concentrations are denoted in square brackets. As a well characterized example, we will take PtdIns(4,5) P_2 , with the biosensor derived from the PH domain from PLCD1 (Table 1). This domain has a well established K_d of 2 μM for binding to PtdIns(4,5) P_2 [3,4,96], and for now we will ignore binding to Ins(1,4,5) P_3 . Considering the cellular [PtdIns(4,5) P_2], McLaughlin has estimated this from first principles to be $\sim 10 \mu\text{M}$ [97], corresponding to empirical estimates ranging from $\sim 4 \mu\text{M}$ in neuroblastoma cells [95] to $\sim 17 \mu\text{M}$ in fibroblasts [98]. Therefore, we can estimate the

fraction of biosensor bound to $\text{PtdIns}(4,5)P_2$ as anything from 50% to 90% (Figure 4A), which grossly fits with what is observed. For example, Figure 4 shows a human neuroblastoma cell line (SH-SY5Y) with approximately 64% of its PH-PLCD1-GFP estimated to be present at the plasma membrane, corresponding to an estimated $[\text{PtdIns}(4,5)P_2]$ of 3.5 μM , in good agreement with estimates for mouse neuroblastomas [95].

Now let's consider a fairly subtle change in $[\text{PtdIns}(4,5)P_2]$: treatment of SH-SY5Y with micromolar wortmannin to inhibit PtdIns 4-kinases, which causes a modest ~15% reduction in $\text{PtdIns}(4,5)P_2$ [99] to ~3 μM . This will cause the cytosolic fraction of PH-PLCD1-GFP to increase from 36%, to 40%, a factor of 10%. How easy would this be to detect? A simple but effective measurement (as performed in Figure 4) is to measure the fluorescence intensity of a region of interest in the cytoplasm. The confocal volume imaged with a high numerical aperture objective effectively excludes membrane fluorescence axial to the focal plane in a typical tissue culture cell several microns thick, so assuming an adequate signal to noise ratio, the 10% increase should be readily detectable. However, precise quantitation of such fluorescence changes is subject to a number of caveats. Firstly, motile cellular structures that exclude the biosensor and occupy a significant fraction of the confocal volume (mitochondria are a good example) can cause fluctuations in the fluorescence recorded from a region of cytosol. Next, many biosensors are small enough to diffuse into the nucleus, so fluorescence increases in the cytosol from newly released probe then equilibrates relatively slowly with the nuclear pool. Finally, cell movement itself can be an issue, especially for extended measurements or when agonists are used that stimulate cytoskeletal dynamics. For these reasons, although small changes can be detected, they are difficult to accurately quantify.

A way to avoid many of the problems with cytosolic measurements is to apply fluorescence microscopy techniques that measure fluorescence intensity specifically of the membrane associated fraction of biosensor. One method is to employ total internal reflection fluorescence (TIRF) microscopy, which selectively illuminates a sub-resolution (typically ~100 nm) layer at the glass water interface. Since this layer encompasses the plasma membrane and relatively little cytosol compared to a confocal or wide-field focal volume, it is exquisitely sensitive [78,92]. Indeed, in our example of wortmannin in SH-SY5Y cells, we expect a change in the fraction of PH-PLCD1-GFP from 64% to 60%, a factor of 6% change in fluorescence. This is well within the range that can be effectively detected by TIRF [78,82].

Another sensitive method is to employ Förster resonance energy transfer (FRET) to detect close proximity of the lipid biosensor with a membrane bound fluorescent protein [100]. This method has the advantage of being applicable to multiple cellular compartments, as opposed to just the plasma membrane with TIRF.

A popular method to boost the measured change in biosensor localization is to record both the membrane-associated and cytosolic fluorescence and calculate the ratio [62,63]. However, this is subject to the same confounds that we list above for cytosolic measurements. Our recent experience with the P4M $\text{PtdIns}4P$ biosensor was that the highly

motile endosome-associated pool prevented measurements from fixed regions of interest of both the membranes and the cytoplasm, since P4M-decorated vesicles rapidly move in and out of such regions. Our solution was to use other fluorescent markers of these compartments to generate dynamic masked regions of interest, and normalize this to the total cellular fluorescence rather than the cytosol. This method captures increases in fluorescence in the cytosol and other membrane compartments when the probe is displaced from one of its resident Golgi, endosomal or plasma membranes [82]. However, it makes estimations of the fraction of biosensor associated with any given compartment extremely difficult.

Up until this point, with our specific example of the PH-PLCD1-GFP PtdIns(4,5) P_2 biosensor, we have ignored binding of the probe to the product of phospholipase C activation, Ins(1,4,5) P_3 . We do so at our peril, since the PH domain binds this inositol phosphatase with sub-micromolar affinity [4,96]. Returning to our example of SH-SY5Y cells (Figure 4), activation of phospholipase C in these cells causes a decrease of approximately 85% of PtdIns(4,5) P_2 mass [99] to an estimated ~ 0.5 μ M, expected to increase the cytosolic fraction of PH-PLCD1-GFP from 36% to 79%. As we can see from Figure 4B & C, the actual increase in PH-PLCD1-GFP is slightly higher, likely reflecting Ins(1,4,5) P_3 competing with residual PtdIns(4,5) P_2 . More generally, the extent to which this competition influences translocation of the probe has been controversial [96,100]. A careful theoretical consideration with empirical measurement revealed that both will influence the translocation of the probe to an extent that depends on the concentrations of PtdIns(4,5) P_2 , Ins(1,4,5) P_3 and-crucially-the biosensor itself [95]. In practice, translocation of PH-PLCD1 cannot be ascribed to a decrease in lipid or increase in inositol phosphate *a priori*; some knowledge of the concentration of these reactants must be available to draw conclusions.

Fortunately, there are simple steps to avoid this issue. The development of a tandem dimer of PLCD1 PH domains [101] shifts the binding towards the much higher concentration of PtdIns(4,5) P_2 in the plane of the membrane relative to Ins(1,4,5) P_3 in the cytosol. Furthermore, the Tubby c-terminal domain (Table 1) exhibits virtually no binding to Ins(1,4,5) P_3 and therefore decreases in membrane binding are directly attributable to decreases in PtdIns(4,5) P_2 [66,87,102]. A new class of biosensor selective for lipid has emerged through the use of PtdIns(4,5) P_2 -regulated ion channels such as $K_{ir}2.1$, which allow rapid and high precision recordings of currents as a proxy for the relative lipid concentration [103,104], although careful selection of channels or their mutants with affinities matching the lipid concentration is necessary for accurate measurement [105], exactly as it is for fluorescent protein biosensors.

Recent efforts have been made to generate a quantitative PtdIns(4,5) P_2 biosensor based on the ENTH domain (Table 1) and fused to an organic fluorophore that exhibits a blue shift upon membrane binding [98]. This elegant approach permits ratiometric imaging of the green cytosolic vs. blue membrane-bound fluorescence as a robust and precise read out of fraction of probe bound, and that is readily calibrated to an *in vitro* binding curve. Although we would argue that this probe is still sensitive to local foldings of the plasma membrane in ruffles and vili etc. that may concentrate membrane relative to cytosol in a confocal volume,

this approach may still prove advantageous when precise quantitative measurements are a must.

Throughout these detailed discussions have focused on PtdIns(4,5) P_2 , but the conclusions are applicable to available biosensors against all the PPIIn. When combined with modern high-sensitivity fluorescence microscopy (or even electrophysiological) techniques, these probes can be extremely sensitive and detect relatively minor changes in the levels of a PPIIn pool in cells (~10%). In fact, it may even be possible to make quantitative estimates of the lipid level, if the stoichiometry of the probe is known, the K_d has been reliably determined and the concentration of biosensor is measured to be substantially lower than that of the PPIIn pool. However, these criteria are rarely met in a typical experiment, and so for the time being, accurate and precise quantitation of PPIIn concentrations with the current stable of biosensors is extremely difficult (and indeed, is not easy with traditional biochemical methods). We urge the reader to exercise caution when interpreting a reduction in signal with any biosensor as evidence of the equivalent quantitative change in lipid abundance.

Cellular distribution of PPIIn

Having discussed in depth the utility of PPIIn binding domains as biosensors for detecting and quantifying PPIIn, we will close with a brief summary of what we have learnt about the lipids' cellular distribution using these probes. As we can see from Table 1, most of the PPIIn show a somewhat restricted cellular distribution and are enriched on specific membranes. However, as we discussed earlier in the article, biosensors have the caveats of only being able to detect pools of PPIIn that accumulate at concentrations $\sim K_d$, and of sometimes exhibiting additional interactions that mislead as to how restricted a PPIIn distribution really is. Indeed, the versatile "co-incidence detection" mechanism for directing protein-PPIIn interaction, as well as emerging roles of lipid transport proteins make it possible that small pools of PPIIn, either highly localized in a specific compartment or else present at concentrations below those detectable with the current biosensors, may exist. For these reasons, we have included this discussion as a separate section to include evidence from more traditional approaches of PPIIn biochemistry and enzymology. Note that we restrict our discussion to the PPIIn present in cytoplasmic membranes; although the generation of PPIIn in the nucleus is an exciting field, they appear to function largely independently of the cytoplasmic pool, and are discussed in an accompanying review by Divecha.

PtdIns

PtdIns is the precursor of all PPIIn yet, ironically, we have the least amount of information about its distribution between the various membranes. PtdIns constitutes about 10–20% (mol %) of total cellular phospholipids depending on the cell or tissue. It is produced in the ER where its synthesizing enzymes, PtdIns synthase (PIS) and CDP-DG synthase (CDS) are located [106]. Early studies based on membrane fractionation in kidney and liver have found PtdIns at the highest level in the Golgi and the ER (7–12 mol % of total phospholipid) although with significant tissue differences [36]. Based on the fact that PtdIns is converted to PtdIns3 P in early endosomes and PtdIns4 P in the Golgi, plasma membrane (PM), early- and late endosomes, it is assumed that PtdIns must be present in all of those membranes. PtdIns is believed to be distributed from its site of synthesis in the ER to other membranes

either by vesicular transport or by non-vesicular lipid transfer proteins, with PtdIns transfer proteins (PITPs) [107,108] thought to be responsible for the latter. It has been postulated that PITPs transfer PtdIns from the ER to the Golgi [109] or from the ER to the PM [110,111]. Interestingly, over-expressed PIS enzyme generates an ER-derived highly mobile membrane compartment that is dependent on PIS enzymatic activity. This may help to deliver PtdIns to various membranes [106]. With all these putative rapid PtdIns distribution mechanisms in place, the steady-state level of PtdIns is quite unpredictable, as the lipid could be made available at any membrane simply based on demand. Curiously, our efforts to obtain indirect information on PtdIns distribution by the use of a PtdIns-specific bacterial PLC enzyme, together with a sensitive diacylglycerol (DG) probe to capture the product, showed that the PLC was able to accumulate DG in the PM, but DG was not detectable in the ER even when the PLC was targeted to the ER membrane [106]. Whether this reflects the rapid metabolism of the generated DG in the ER, the inability of the DG probe to detect DG in ER membranes or a true low steady-state level of PtdIns in the site of its synthesis remains to be determined. However, studies on red blood cell membranes showed the presence of substantial amount of PtdIns [112,113], which together with the above results strongly argues that the PM does contain PtdIns in significant amounts.

PtdIns4P

This lipid constitutes about 2–5% of total PtdIns in a typical mammalian cell [95,99]. PtdIns4P has been clearly recognized a long time ago as the precursor of plasma membrane PtdIns(4,5) P_2 , so there was little doubt that it must be present in the PM. It came then as a surprise that none of the cloned mammalian PI4Ks are particularly PM localized; instead, they are found enriched in the Golgi and several endosomal compartments (except for the yeast Stt4p that appears PM localized [114]) [115]. However, the existence of a PM pool of PtdIns4P was indicated by the fact that the PIP5Ks that use this lipid were associated with the PM. The presence of PtdIns4P in these compartments was then confirmed initially by immuno-staining [116] and most recently by the new unbiased P4M probe from the *Legionella* protein SidM [82]. Based on these measurements and given the estimated fraction of the PM of all the cellular membranes (estimated 1–14% of total cell surface) [117], a substantial fraction of the total PtdIns4P must be present in the PM. This conclusion is also supported by cell fractionation studies using [^3H]-inositol labeled lipids [118]. It is worth noting that metabolic labeling with ^{32}P -phosphate labels PtdIns4P most rapidly, but this pool with high metabolic turnover does not represent PtdIns4P across the whole cell. Based on inhibitor studies the high PtdIns4P labeling likely over-represents the PM pool of PtdIns4P that is generated by PI4KA [119]. PtdIns4P in various compartments is made by distinct PI4K enzymes: the Golgi pool is primarily produced by PI4KB [120] with help from PI4K2A and B, especially in the Tgn [121,122], whereas the early and late endosomal PtdIns4P is made by the type II PI4Ks [82], although a lysosomal function of PI4KB has also been reported [123]. The PM pool is mostly generated by PI4KA but based on studies in red blood cell membranes, type II PI4Ks probably also make a contribution. Although PtdIns4P is not detected in the ER, the ER localization of PI4KA and its pivotal role in the formation of the probably ER-derived viral replication “membranous web” in hepatitis C infected cells [124], suggest that PtdIns4P is important in the ER possibly at ER exit sites or the ERGIC compartment [125].

PtdIns3P

In mammalian cells, PtdIns3P is present in a low amount representing about 5–10% of PtdIns4P [126]. In yeast, however, the levels of PtdIns3P and PtdIns4P are comparable [127]. There is relatively little ambiguity that PtdIns3P is found primarily in early endosomes, since several specific and high affinity biosensors are available for PtdIns3P (Table 1). To our knowledge, no study has directly assessed whether this lipid is sufficient to drive localization of these probes. That said, the consistent early-endosomal localization achieved with these structurally unrelated domains strongly imply that they correctly report this as the major pool present in cells. An EM analysis using a recombinant 2xFYVE domain also showed concentration of the lipid associated with the internal vesicular membranes of multivesicular bodies (MVBs) [60]. PtdIns3P has a high turnover rate requiring constitutive PtdIns phosphorylation, as indicated by the rapid disappearance of the PtdIns3P-sensing lipid probes after inhibition of the PI 3-kinases [60]

Notably, a recent cell fractionation study has found the majority of PtdIns3P associated with @COP positive ER/Golgi membranes [118]. Whether this reflects a redistribution of the lipid during fractionation or the inability of the lipid probes to locate the lipid in this compartment, is yet to be seen. Importantly, several studies have shown the essential role of the Class III PI3K, hVps34 in autophagosomal membrane formation [128]. This is an important point to recognize as the origin of these autophagosomal membranes are believed to be the ER with the help from mitochondria [129], but not particularly from endosomes. This finding would agree with the lipid being found in the ER. The bigger question related to PtdIns3P, however, is which enzyme(s) are responsible for its production. It is clear that hVps34 is a major source of PtdIns3P in early endosomes and in autophagosomes, but there is increasing evidence that Class II PI3Ks may also generate this lipid in specific endosomal compartments [130]. Again, the conclusion that can be drawn from all of these reports is that PtdIns3P levels may not have to be very high (detectable by our current probes) at all places where the lipid plays an important role by interaction with downstream effector proteins.

PtdIns5P

This is one of the most enigmatic members of the PPIIn family. It is estimated that its levels are about 1 % of PtdIns4P in mammalian cells [118,126] and its localization is highly debated (Figure 1). The importance of this lipid is recognized, as it serves as the precursor of the type II PIP kinases [131]. Although the exact role of these enzymes is still being explored, their possible nuclear functions [132] and connections to Akt and mTOR signaling [59,133,134] and carcinogenesis [135,136] makes PtdIns5P a highly desirable target to investigate, although as we discussed earlier, no probe is available that can detect the scant endogenous level. Cell fractionation studies have found the highest amount of PtdIns5P in PM fractions [118,137] and an enrichment of the lipid in ER/Golgi fractions [118] or in early endosomes [133]. Enzymatically, PtdIns5P can be formed directly by PtdIns phosphorylation by the PIKfyve enzyme [138], or via dephosphorylation of PtdIns(3,5)P₂ by myotubularin phosphatases [126]. Which of these pathways are more important in the cell is still highly debated. It is now generally assumed that type II PIP kinases are more important in controlling PtdIns5P levels than generating PtdIns(4,5)P₂, the majority of which is made via the canonical PtdIns4P phosphorylation route [137].

PtdIns(4,5) P_2

This is the most abundant phosphorylated PPIIn, amounting to 2–5 % of total PtdIns [95,99]. Notably, yeast and plants contain significantly lower levels of PtdIns(4,5) P_2 and more PtdIns4 P than mammalian cells [139,140]. Most of the PtdIns(4,5) P_2 is located in the PM as shown either by expressed fluorescent lipid reporters [64] or by anti-PtdIns(4,5) P_2 antibodies or recombinant PH domains [61]. EM analysis of PtdIns(4,5) P_2 distribution assessed by a GST-fused PLCTM₁PH domain also showed small amounts in the Golgi and endosomes [53]. However, biochemical evidence suggests that PtdIns(4,5) P_2 can regulate Golgi-localized effectors such as various Arf1 GAPs [42,43], making a small amount of PtdIns(4,5) P_2 in the Golgi feasible. An intracellular role of PtdIns(4,5) P_2 has also been shown for endolysosomal processing of EGF receptors and E-cadherin [141,142], although many 5-phosphatases are important during endocytosis at the plasma membrane to strip PtdIns(4,5) P_2 before conversion to 3 phosphorylated PPIIn on the endosomes [143].

As the number of PM-bound effectors regulated by PtdIns(4,5) P_2 is increasing [2], the question arises: how is the lipid able to control so many effectors seemingly independently in the same membrane? Several theories have been entertained, some suggesting the existence of PtdIns(4,5) P_2 enriched membrane domains, related or unrelated to lipid rafts, the existence of lateral diffusion barriers, or the localized channeling of the lipids by the kinases to specific molecular effectors etc. Experimental evidence for these mechanisms are not easy to obtain. The other hard-to-explain findings are related to the relationship between the PM-localized PtdIns4 P and PtdIns(4,5) P_2 . It appears that cells can maintain their basal PtdIns(4,5) P_2 levels at a wide range of PM PtdIns4 P [38,119] even though it is clear that PM PtdIns4 P is an obligate intermediate for PtdIns(4,5) P_2 synthesis. How cells sense their PM PtdIns(4,5) P_2 levels and what homeostatic mechanisms keep it within a range are exciting questions for future studies. What is clear at present is that the major pool of PtdIns(4,5) P_2 resides and is indeed metabolized at the PM, where it is required to control an assortment of transport, signaling and cytoskeletal processes.

PtdIns(3,4) P_2

This is also a minor lipid with basal levels at detection limits but it can substantially increase upon activation of PI3Ks. The largest elevation in PtdIns(3,4) P_2 is achieved by treatment with H₂O₂, which, through inhibition of PTEN and probably the PtdIns(3,4) P_2 phosphatases, INPP4A/B, slowly converts almost the entire PtdIns(4,5) P_2 pool to PtdIns(3,4) P_2 [144,145]. However, in a normal stimulated cell PtdIns(3,4) P_2 levels reach only 2–5 % of those of PtdIns(4,5) P_2 [146]. Because the main source of PtdIns(3,4) P_2 is PtdIns(3,4,5) P_3 made by Class I PI3Ks in the plasma membrane and dephosphorylated by SHIP1/2 5-phosphatases, this lipid was assumed to be localized to the PM. This was confirmed with the use of the TAPP1 PH domain as a probe in live [147] or fixed cells [146] and the lipid was found especially enriched in lamellopodia [148,149]. EM analysis using the TAPP1 PH domain as a probe found some of the lipid in the ER and endosomes, especially in the lumen of MVBs [57]. A recent study unveiled a role of PtdIns(3,4) P_2 in controlling the maturation of clathrin-coated vesicles and showed the presence of the lipid in late stages of endocytic intermediates [143]. Therefore, the current view is that

PtdIns(3,4) P_2 is most likely generated at the PM, but may persist through endocytotic events and therefore be trafficked to distant membrane compartments.

PtdIns(3,5) P_2

This minor PPIIn is critical for the proper sorting of cargoes in the late endosomal stage and during formation of the internal vesicles in MVBs. PtdIns(3,5) P_2 levels in yeast are ~50% of those of PtdIns(4,5) P_2 [140,150]. Their amount is significantly lower in mammalian cells (1% of PtdIns(4,5) P_2 [126,138]) making it the PPIIn of lowest abundance. Because of the lack of appropriate tools to visualize them, the localization of this lipid species has been deduced only from functional studies and from the localization of the enzymes that make and degrade this lipid species. A recent study has reported the localization of PtdIns(3,5) P_2 to late-endosomes/lysosomes using a tandem sequence from the N-terminus of the mucolipin channel, TRPML1 as a probe [83]. However, we have been unable to substantiate these claims (see above). Immunostaining with an anti PI(3,5) P_2 antibody showed a mitochondrial staining [151] but based on what we know about PI(3,5) P_2 physiology this localization is highly questionable. Therefore we are still searching for an appropriate tool to find this lipid inside the cell.

PtdIns(3,4,5) P_3

This minor lipid has generated a lot of attention because of its oncogenic potential. It represents about 1–5% of PtdIns(4,5) P_2 with almost undetectable levels in quiescent cells that rise upon activation of Class I PI3Ks [152]. PtdIns(3,4,5) P_2 is generated primarily in the plasma membrane and that is where it has been detected either in live cells using PH-domain-based reporters (See table 1) or by antibody staining [153]. PtdIns(3,4,5) P_3 shows significant enrichment within the plasma membrane in the front of migrating cells pointing to the chemotactic source but also in active membrane domains such as lamellopodia [154]. Perhaps the most striking lateral segregation and concentration of PtdIns(3,4,5) P_3 is seen in the phagocytic cup [155], where the diffusion of the lipid must be significantly curtailed to maintain a localized enrichment. Intracellular PtdIns(3,4,5) P_3 has not been detected widely, but a recent study showed that PI3K γ associated with its p101 regulatory subunit generates a PtdIns(3,4,5) P_3 pool in the PM that undergoes endocytosis whereas the PtdIns(3,4,5) P_3 pool produced by the p84/p110 γ complex remains in the PM [156].

Perspective

The Biology of PPIIn has been active since the 1940s, with our appreciation of their protein interactions exploding in the 1990s, as we saw in the first section of this review. Although many principles of protein-PPIIn interaction are understood, often with atomic resolution, new examples continue to be discovered and we are far from genomic annotation with a completed list of these interactions. Recent discoveries of the coupling of PPIIn metabolism with lipid transfer [50,80,157] and host-pathogen interaction (see the review by Cossart in this issue) underscore how new paradigms continue to emerge.

In light of these continuing advances, it is clear there is still plenty of utility for the current toolbox of PPIIn biosensors (table 1), and indeed for the development of improved versions.

Despite the limitations discussed in this review, there are currently excellent tools for the detection of most PPI in living cells. The current lack of proven probes for PtdIns(3,5)P₂, PtdIns5P and PtdIns are notable gaps and are priorities for the future. Another key question is how cells co-ordinate these legion protein-PPI interactions, and couple this to feedback control of PPI metabolism. The development of new tools that can probe these interactions at the molecular scale will be key to finding answers. We are particularly excited by recent advances in the fields of liquid-chromatography mass spectrometry [85], “imaging” mass spectrometry [86] and the field of super-resolution light microscopy [158] as being poised to produce new insights in this area. In short, we look forward to yet more discoveries in biosensor development driving our understanding of basic PPI biology and its impact on human disease.

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Highlights

- Polyphosphoinositides (PPI_n) binding domains come from many families, with a broad spectrum of affinity and selectivity
- Their regulation by lipids can take several forms, from simple membrane targeting to specific allosteric regulation
- High affinity, specific binding domains can make excellent probes to study the lipids in living cells
- The usefulness of the probes depends on their specificity, and whether the target lipid is necessary and sufficient for targeting
- Quantitation with such probes requires careful consideration of binding affinity
- PPI_n binding domains have been instrumental in defining the cellular distribution of the various PPI_n on plasma, endocytic and late secretory membranes.

Figure 1. PPIIn and their cellular distribution

The structure of phosphatidylinositol (PtdIns) is shown with the hydroxyls 3–5 available for reversible phosphorylation indicated. The cartoon shows a generic mammalian cell with the major PPIIn conversion reactions indicated in their native membranes. Note that PtdIns synthesis occurs in the endoplasmic reticulum (ER), from whence it is known to be transferred to the plasma membrane or Golgi as depicted.

Figure 2. Five functional principles underlying PPIIn-regulated protein function

(i) high affinity binding between protein and PPIIn drives membrane binding (ii) co-incident binding between a protein, PPIIn and a tertiary determinant (in this case a small G-protein, but it may also be a physical determinant such as membrane curvature) provide the avidity necessary for membrane binding. (iii) Polyvalent clustering of PPIIn through non-specific electrostatic interactions with the polybasic region of an amphilic peptide motif contributes to membrane targeting at anionic lipid-rich membrane (iv) binding of PPIIn induces a conformational change in a binding protein. (v) PPIIn interaction with a transfer protein facilitates lipid transfer.

Figure 3. specificity of two PtdIns(4,5) P_2 biosensors in cells

Depletion of PtdIns(4,5) P_2 at the PM causes release of Plcd4-PH-GFP, showing the dependence on this lipid for probe localization. Alternatively, driving ectopic synthesis of PtdIns(4,5) P_2 at Rab7-labelled membranes is sufficient to recruit the PtdIns(4,5) P_2 biosensors PH-Plcd4-GFP and Tubby_c-GFP. The indicated biosensors fused to GFP were expressed in COS-7 cells with either an FRB-PM motif (N-terminal peptide from Lyn kinase) and FKBP fused to the INPP5E 5-phosphatase, or an FRB-fused Rab7 and an FKBP-fused PIP5K. Addition of rapamycin induced dimerization of FRB and FKBP, leading to recruitment of the enzyme to the labelled compartment and depletion or synthesis of PtdIns(4,5) P_2 , respectively. Insets show enlarged view of the boxed region.

Figure 4. quantitative aspects of a PtdIns(4,5) P_2 biosensor

A. The fraction of PH-PLCD1-GFP expected to be membrane bound in a cell with varying PtdIns(4,5) P_2 concentrations. For SH-SY5Y cells the fraction bound (~64%, red line) was estimated after treating cells with ionomycin to cause complete release of the probe into the cytosol, giving the estimated [PtdIns(4,5) P_2] = 3.5 μ M. **B.** Three SH-SY5Y cells expressing PH-PLCD1-GFP, either at rest or after activation of endogenous cholinergic M3 receptors with carbachol. **C.** Carbachol treatment is known to induce ~85% depletion of PtdIns(4,5) P_2 , and the theoretical and experimentally-measured change in cytosolic fraction of biosensor is shown. See text for details.

Table 1

PPIn biosensors and their properties

The seven PPIn, along with some of the more commonly used biosensors that are expressed as fusions with fluorescent-proteins.

PPIn	Biosensor	localization	K_d	PPIn specific?	PPIn dependent?	PPIn sufficient?
PtdIns3P	FYVE-Hrsx2	EE	2.5 μ M [159](monomer)	✓ [8,9]	✓ [60]	?
	FYVE-EEA1	EE	45 nM [160]	✓ [8,9]	✓ [8,9]	?
	PX-p40phox	EE	5 μ M (diC4-PtdIns3P) [161]	✓ [11,12]	✓ [11,12]	?
PtdIns(3,5)P ₂	ML1-Nx2	LE	?	✓ [83]	?	?
PtdIns4P	PH-OSBP	Golgi/PM*	3.5 μ M [71]	✗ Binds PtdIns(4,5)P ₂ [71]	✓ [72,76,77]	✗ Golgi (Arf1-dependent) [72, 71,75,76] ✓ PM [75,78]
	PH-FAPP1	Golgi	18.6 μ M [71]	✗ Binds PtdIns(4,5)P ₂ [71]	✓ [72,76,77]	✗ Arf1-dependent [71,75,77]
	PH-Osh2×2	PM	?	✗ Binds PtdIns(4,5)P ₂ [72]	✗ Binds PtdIns(4,5)P ₂ [38,79]	✗ Cannot detect Golgi-PtdIns4P [72]
	P4M-SidM	Golgi/PM/LE	~ 1 μ M (1/50 full length SidM at 18.2 nM) [32,81]	✓ [32]	✓ [82]	✓ [82]
PtdIns(4,5)P ₂	PH-PLCD1	PM	2 μ M [3,4,96]	✗ Binds Ins(1,4,5)P ₃ [3,4,96]	✓ [162,163]	✓ [82]
	PH-Plcd4	PM	? but > PH-PLCD1 [164]	✗ Binds Ins(1,4,5)P ₃ [164]	✓ Fig. 4	✓ Fig. 3
	Tubbyc	PM	?	✗ Binds PtdIns(3,4)P ₂ /PtdIns(3,4,5)P ₃ [66]	✓ [87]	✓ Fig. 3
	ENTH/ANTH	PM	5 μ M [14,15]	✓ [14,15]	✓ [98]	?
PtdIns5P	PHD-Ing2×3	Nucleus/PM*	?	✗ Binds PtdIns3P [68]	✓ [68]	✓ PM [68] ? nucleus [68]
PtdIns(3,4,5)P ₃	PH-Akt	PM*	590 nM [165]	✗ Binds PtdIns(3,4)P ₂ and InsP ₄ [165,166]	✓ [167–169]	?
	PH-Btk	PM*	80 nM [165]	✗ Binds Ins(1,3,4,5)P ₄ [89,165,170,171]	✓ [165,172]	?
	GRP1-PH	PM*	170 nM [165]	✗ Binds Ins(1,3,4,5)P ₄ [22,165]	✓ [22,65]	✗ Requires Arf/Arf [26–28]
PtdIns(3,4)P ₂	TAPP1-PH-CT	PM*	80 nM [165]	✓ [174]	✓ [147]	✓ [147]

Localization: EE (Early endosome), LE (late endo/lysosomes), PM (plasma membrane) and * denotes a localization viewed after stimulation of lipid synthesis. The dissociation constant K_d is reported as binding to the PPIn in a membrane unless indicated. Satisfaction of three criteria that define the specificity of the biosensors is denoted by ✓ and failure to do so by ✗ -see text for details.