Multiple Pools of Phosphatidylinositol 4-Phosphate Detected Using the Pleckstrin Homology Domain of Osh2p*

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Pleckstrin homology (PH) domains from three families of lipid-binding proteins target the Golgi (12, 13, 18–21). In addition, clathrin adaptor AP1 complex was also shown to interact with PtdIns(4)P on the Golgi (22). Yeast also harbor a third PtdIns 4-kinase, Lsb6p, which is in the type II family, and has no known function (23).

The question posed by the presence of these different PtdIns 4-kinases is how might proteins detect one specific pool of PtdIns(4)P among the multiple organelles that have active PtdIns 4-kinases? One plausible explanation supported by previous observations with the PH domain of OSBP is that proteins cannot target a specific compartment by virtue of PtdIns(4)P alone, but achieve restricted targeting by binding a membrane lipid in combination with other membrane receptors (12, 24). OSBP is a peripheral membrane protein that targets the late Golgi solely by virtue of a PH domain that binds to PtdIns(4)P and PtdIns(4,5)P_2 with equal affinity (12, 18). In addition, PH^{Osh2} binds a Golgi-restricted receptor dependent on the small GTPase Arflp (12), and has recently been shown to bind weakly to ARF1 (21).

Here, we focus on two PH domains from yeast homologues of OSBP: Osh1 and Osh2. Whereas PH^{Osh1} targets Golgi membranes, PH^{Osh2}, a closely related domain (71% identical and 91% homologous) targets membranes weakly (25). We have identified the site within PH^{Osh2} that binds a second mem-
branl receptor, and developed a tool based on PHOsh2 that lacks the second site and so detects pools of 4-phosphorylated PtdIns in an unbiased manner. Using PHOsh2 we demonstrate the physical separation of the two pools of PtdIns(4,5)P2 dependent on Sac1p and Piki1p, on the plasma membrane and Golgi, respectively. We then compare PHOsh2 to other PH domains that bind PtdInsPs within this NH2-terminal region (28, 29). In contrast, if PHOsh2 had no effect on targeting (Fig. 1), whereas PHOsh2 shows barely discernible membrane targeting in yeast (Fig. 1B) and fibroblasts (data not shown). This differs from a recent study showing stronger Golgi targeting by monomeric PHOsh2, although in that case a basic patch adjacent to the core domain sequence was also included, which may alter targeting (27).

The portion of primary sequence responsible for the difference in targeting between PHOsh1 and PHOsh2 was mapped using a series of chimeras in which portions of the Osh1p sequence were exchanged for the homologous Osh2p sequence. For this purpose, the PH domain (105 amino acids in Osh1p) was divided into five approximately equal segments, and chimeras were made that contained mixtures of Osh1p and Osh2p sequences (see "Experimental Procedures"). Substitution of the NH2-terminal three-fifths of PHOsh1 with the equivalent region of PHOsh2 had no effect on targeting (Fig. 1C), despite the almost universal location of the binding site in PH domains for PtdInsPs within this NH2-terminal region (28, 29). In contrast, inclusion of the next (i.e.) fourth) segment of PHOsh1 inhibited membrane localization (Fig. 1D). The same requirement for the fourth segment of PHOsh1 was seen for Golgi targeting in mammalian COS cell fibroblasts (data not shown).

Within the critical fourth segment of 22 residues, PHOsh1 differs from PHOsh2 at 8 positions. By further dissection of this segment, a single residue, histidine at position 79 in PHOsh1 compared with arginine at position 80 in PHOsh2, was shown to be critical for targeting (Table II). To confirm the role of this single residue, arginine was introduced at position 79 of PHOsh1, in which it strongly inhibited Golgi targeting (Fig. 1E). Introduction of histidine at the same site in PHOsh2 induced a striking gain of Golgi targeting (Fig. 1F).

Modeling of the sequence of PHOsh1 on known PH domain structures (28, 29) shows that this residue lies in the middle of the β strand. In all known structures, this residue points away from the core of the domain on a side of the molecule at some distance from the membrane interface, assuming that this is defined by the site that binds PtdInsPis (Fig. 2). These results therefore indicate that the second site is unlikely to interact with a membrane lipid receptor, but is more likely to be a protein.

**RESULTS**

**Identification of the Second Site in PHOsh1 for Golgi Targeting—** PHOsh1 targets Golgi membranes in yeast (Fig. 1A) and mammalian fibroblasts (24, 25), whereas PHOsh2 shows barely discernible membrane targeting in yeast (Fig. 1B) and fibroblasts (data not shown). This differs from a recent study showing stronger Golgi targeting by monomeric PHOsh2, although in that case a basic patch adjacent to the core domain sequence was also included, which may alter targeting (27).

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**PHOsh1 Binds Both PtdIns(4)P and PtdIns(4,5)P2—** If PHOsh2

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**EXPERIMENTAL PROCEDURES**

**Plasmids—** PH domains were defined as follows (alternative gene names together with accession numbers are given in brackets, followed by starting and ending residues, identified using the three letter amino acid code): Osh1p (YAR492W/YAR404W, YA41177) Glu79-Arg52; Osh2p (YDL019C, NP_010265) Ser286-Lys391; phospholipase C (CERT, previously called Goodpasture's antigen-binding protein) and phosphoinositol 4-phosphate adaptor protein 1 (FAPP1) as before (12).

**TABLE I**

| Plasmids | Ref. or source |
|----------|---------------|
| PHO5      | pRS406 Integrates at URA3 | Ref. 48 |
| PHO5      | pRS416 CEN URA3 (low copy) | Ref. 48 |
| pTL31     | pRS406 PHOS prom., GFP, myc, PHOsh1 (105 aa = 280–384 aa) |
| pTL32     | pRS406 PHOS prom., GFP, myc, PHOsh1 (99 aa = 87–185 aa) |
| pTL33     | pRS406 PHOS prom., GFP, myc, PHOsh1 (122 aa = 1–122 aa) |
| pTL34     | pRS406 PHOS prom., GFP, myc, PHOsh1 (98 aa = 1–98 aa) |
| pTL32     | pRS416 PHOS prom., GFP, PHOsh2 (169 aa = 256–424 aa), GFP | Ref. 25 |
| pTL502    | pRS406 PHOS prom., GFP, myc, PHOsh2 (106 aa = 286–391 aa) |
| pTL503    | pRS406 PHOS prom., GFP, myc, PHOsh2 (aa 1–61), PHOsh1 (aa 61–105) |
| pTL504    | pRS406 PHOS prom., GFP, myc, PHOsh2 (aa 61–82), PHOsh1 (aa 82–105) |
| pTL505    | pRS406 PHOS prom., GFP, myc, PHOsh2 (aa 61–70), PHOsh1 (aa 61–70) |
| pTL506    | pRS406 PHOS prom., GFP, myc, PHOsh2 (aa 71–82), PHOsh1 (aa 71–82) |
| pTL507    | pRS406 PHOS prom., GFP, myc, PHOsh2 (aa 71–77), PHOsh1 (aa 72–83) |
| pTL508    | pRS406 PHOS prom., GFP, myc, PHOsh2 (aa 71–77), PHOsh1 (aa 72–83) |
| pTL509    | pRS406 PHOS prom., GFP, myc, PHOsh2 (aa 71–77), PHOsh1 (aa 72–83) |
| pTL510    | pTL502 PHOsh1 (aa 71–77), PHOsh1 (aa 72–83) |
| pTL511    | pTL502 PHOsh1 (aa 71–77), PHOsh1 (aa 72–83) |
| pTL512    | pTL502 PHOsh1 (aa 71–77), PHOsh1 (aa 72–83) |
| pTL513    | pTL511 carrying mutations R307E R308H in both copies of PHOsh1 |
| pTL514    | pTL333 carrying mutation R43E in PHCERT |

**a** aa, amino acid.
is fully capable of membrane targeting, but just lacks a second site, it should bind PtdInsPs similarly to PHOsh1. To test this, we compared the binding of GFP-tagged PH domains to liposomes containing 2.5% PtdIns(4)P or PtdIns(4,5)P2 (12) (Fig. 3). As controls, PHOsh1 showed its well documented absolute preference for PtdIns(4,5)P2 over PtdIns(4)P, and PHAPF1 bound to both lipids, with a slight preference for PtdIns(4)P (Fig. 3). PHOsh2 and PHOsh1 showed that PHOsh1 bound PtdIns(4,5)P2 with almost identical affinity, greater than that of PHAPF1 (Fig. 3A); PHOsh2 bound PtdIns(4)P more strongly than PHOsh1 (~2-fold more) (Fig. 3B). Overall, these experiments show that PHOsh2 binds PtdIns(4)P and PtdIns(4,5)P2 with affinity similar to other PH domains that target membranes efficiently, despite its very weak membrane targeting in vivo.

A PHOsh2 Dimer Targets Two Pools of 4-Phosphorylated PtdInsPs in Growing Cells—The above data show that PHOsh2 binds 4-phosphorylated PtdInsPs, but it differs from closely related PH domains by the absence of a second site. Therefore, we reasoned that if a reporter construct could be made to target membranes dependent primarily on PHOsh2, it would reveal the overall distribution of PtdInsPs in growing cells, with little cytoplasmic background (Fig. 4A). GFP-PHOsh2-dimer expressed from either a relatively weak promoter (OSH1), or a rapidly inducible promoter (GAL1) produced the same dual localization (data not shown), indicating that it is independent of duration and level of expression. Although the distribution of GFP-PHOsh2-dimer became diffuse after fixation (data not shown), partial co-localization was found in live cells with punctae stained by FM4-64, which traverses the endocytic pathway and also enters late Golgi compartments prior to being sorted back to the cell surface (Fig. 4B) (31). Therefore, we conclude that the punctate structures are late Golgi and/or early endosomes. In addition, the dual targeting of PHOsh2 implies either that it interacts with two different ligands, or alternatively that a single ligand is present in two sites. An indication that both localizations of GFP-PHOsh2-dimer are PtdInsP-dependent was gained using a dimer in which two conserved positive residues of both PH domain sequences contained mutations that are known to prevent the PtdInsP binding site of the PH domains (12), which had an entirely diffuse localization (Fig. 4C).

To test whether Pik1p plays a role in the localization of PHOsh2, as for PHOSBP (12), GFP-PHOsh2 dimer was expressed in a strain carrying a conditional pik1 allele. Shift to the non-permissive temperature caused a rapid and almost complete loss of internal punctate localization in the mutant cells, with only a slight effect seen in wild-type cells undergoing the same temperature shift (Fig. 5A). By comparison, PHOSBP is only incompletely delocalized under these conditions (12). Therefore, PtdIns(4)P produced by Pik1p is likely to be the Golgi ligand for PHOsh2. Next, we sought to identify the plasma membrane ligand for PHOsh2. Initially we looked at the role of PtdIns(4,5)P2 by expressing GFP-PHOsh2 dimer in cells carrying the mss4-2ts allele. Little effect on the plasma membrane localization was detected at the non-permissive temperature under conditions where PtdIns(4,5)P2 levels are known to fall (Fig. 5B) (12). This result does not rule out a role for PtdIns(4,5)P2 at the plasma membrane, but indicates that there must be an additional ligand present when PtdIns(4,5)P2 production is blocked.

We next tested whether the other essential PtdIns(4)-kinase, Stt4p, was responsible for synthesizing a pool of PtdInsP on the plasma membrane that is recognized by PHOsh2. In temperature-sensitive strains carrying the mutant allele stt4-4ts, the localization of the PHOsh2 dimer was altered, with much reduced plasma membrane localization even at the permissive temperature (data not shown). Plasma membrane targeting was almost completely lost at the non-permissive temperature, whereas punctate localization was preserved (Fig. 5C), and these effects were not seen in wild-type controls (Fig. 5C), indicating that Stt4p synthesizes the 4-phosphorylated PtdIns that PHOsh2 binds to the plasma membrane. We also examined the role of the single PtdIns 3-kinase, Vps34, because it is possible that PHOsh2 binds either PtdIns(3)P or PtdIns(3,5)P2. However, localization of the GFP-PHOsh2 dimer was unaffected in Δvps34 cells (data not shown). The combination of this result and the complete loss of punctate localization with the pik1 mutation indicates that the punctate localization of PHOsh2 is not mediated by PtdIns(3)P. Overall, these results show that each of the dual localizations of this PH domain is sensitive to inactivation of a different essential PtdIns 4-kinase, demonstrating that PHOsh2 interacts with two separate pools of 4-phosphorylated PtdInsPs.

PHOsh2 as a Reporter for Altered PtdInsPs Levels in Δsac1 Cells—The results above indicate that PHOsh2 is an unbiased reporter of PtdInsPs and PtdIns(4,5)P2, so we next examined how its distribution was affected by a well characterized mutation that alters PtdInsPs levels. Sac1p is one of five PtdInsP phosphatases in yeast, and its inactivation leads to a large increase in cellular PtdInsPs, up to 10-fold, together with a fall in PtdIns(4,5)P2 (16, 32). To determine the effect of these
A PH Domain That Binds Multiple Pools of PtdIns(4)P

GFP-tagged PH\textsuperscript{Osh1} constructs that varied only in the sequences in the fourth out of five segments of PH\textsuperscript{Osh1} (residues 61–82) were scored for their punctate localization. Introduction of the Osh2p sequence (construct 1) made 8 changes and rendered membrane targeting only weakly detectable (w). While the NH\textsubscript{2}-terminal 3 variant residues had little effect on targeting (construct 2), the COOH-terminal 5 variant residues were responsible for the majority of targeting (construct 3). Introduction of the first 4 of these 5 changes had no effect on targeting (construct 4), indicating an important role for the last variant residue: His\textsuperscript{79} in PH\textsuperscript{Osh1} (Arg\textsuperscript{80} in PH\textsuperscript{Osh2}).

| Construct | Plasmid | Sequence between residues 61 and 82 of PH\textsuperscript{Osh1} | No. changes, cf. Osh1p | Punctate |
|-----------|---------|---------------------------------------------------------------|------------------------|----------|
| Osh1p     | pTL331  | SEKLFKEIIGGNGVirWHLKG                                        | 0                      | ++       |
| 1         | pTL504  | SEKLFKEIIGGNGVirWHLKG                                        | 8                      | w        |
| 2         | pTL505  | SEKLFKEIIGGNGVirWHLKG                                        | 3                      | +        |
| 3         | pTL506  | SEKLFKEIIGGNGVirWHLKG                                        | 5                      | w        |
| 4         | pTL507  | SEKLFKEIIGGNGVirWHLKG                                        | 4                      | ++       |

**TABLE II**

Analysis of the section of PH\textsuperscript{Osh1} that determines Golgi targeting

PH domains that bind PtdIns(4)P. GFP-PH\textsuperscript{Osh1} did not target the enlarged pool of PtdIns(4)P at the plasma membrane, but remained largely punctate (Fig. 6C). This is further evidence that PH\textsuperscript{Osh1} does not target membranes solely because they are rich in PtdIns(4)P. We next examined the effect of \( \Delta sac1 \) on the distribution of other Golgi-targeted PH domains, using sequences from OSBP, FAPP1, and CERT (previously called Goodpasture’s antigen-binding protein). Like PH\textsuperscript{Osh1}, these target the Golgi by interacting with both PtdIns(4)P and a second site interaction. However, GFP-PH\textsuperscript{CERT} was notably different, being targeted to the nuclear envelope and to peripheral patches, characteristic of the yeast ER (Fig. 6F). To check whether this targeting was caused by binding to PtdInsPs, we next expressed a mutated version of this construct, in which the critical basic residue for PtdInsP binding was mutated to an arginine. GFP-PH\textsuperscript{CERT}(R43E) was entirely diffuse, with no ER or punctate localization (Fig. 6G). On close inspection, all three of PH\textsuperscript{Osh1}, PH\textsuperscript{OSBP}, and PH\textsuperscript{FAPP1} showed much weaker targeting to the nuclear envelope and periphery (Fig. 6). These results suggest that some PtdIns(4)P that accumulates in \( \Delta sac1 \) cells is specifically targeted by PH\textsuperscript{CERT}, possibly because of a unique second site interaction.

**DISCUSSION**

PH domains are sequences usually 100–120 amino acids in length that have a characteristic fold of a seven-stranded $\beta$-sheet rich in PtdIns(4)P. We next examined the effect of \( \Delta sac1 \) on the distribution of other Golgi-targeted PH domains, using sequences from OSBP, FAPP1, and CERT (previously called Goodpasture’s antigen-binding protein). Like PH\textsuperscript{Osh1}, these target the Golgi by interacting with both PtdIns(4)P and a second site interaction. However, GFP-PH\textsuperscript{CERT} was notably different, being targeted to the nuclear envelope and to peripheral patches, characteristic of the yeast ER (Fig. 6F). To check whether this targeting was caused by binding to PtdInsPs, we next expressed a mutated version of this construct, in which the critical basic residue for PtdInsP binding was mutated to an arginine. GFP-PH\textsuperscript{CERT}(R43E) was entirely diffuse, with no ER or punctate localization (Fig. 6G). On close inspection, all three of PH\textsuperscript{Osh1}, PH\textsuperscript{OSBP}, and PH\textsuperscript{FAPP1} showed much weaker targeting to the nuclear envelope and periphery (Fig. 6). These results suggest that some PtdIns(4)P that accumulates in \( \Delta sac1 \) cells is specifically targeted by PH\textsuperscript{CERT}, possibly because of a unique second site interaction.

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sandwich closed off at one side by an amphipathic α helix (28, 29, 33). PH domains have been found to interact with a variety of different ligands, using sites that are somewhat conserved for each purpose. The α helix can interact with WD40 proteins including Gβγ (34), and differing parts of the α helix of PH domains in guanine nucleotide exchange factors bind the neighboring catalytic Dbl homology domain (35). Finally, the best characterized interaction of PH domains arises from the formation of a pocket by loops β1-β2 and β3-β4 to bind PtdIns(4)Ps. Lipid specificity is determined by a combination of basic residues lining the pocket and a generally positive electrostatic charge on this face of the domain. Whereas a few PH domains, in particular those that bind PtdIns(3,4,5)Ps, show high affinity binding for just a single PtdInsP, most PH domains, including PHFAPP1 and PHOSBP with ARF1 in mammalian cells (the equivalent of yeast Arf1p). On this basis, one would therefore

**FIG. 4. Dual localization of PHOsh2 dimer.** A, PHOsh2 was expressed as a tandemly repeated dimer of PHOsh2 extended ~30 amino acids at each end (total of 169 amino acids) bracketed by GFP (using pTL511). The dimer showed a dual localization: at the plasma membrane particularly enriched in small buds; and in numerous punctate structures often seen at the bud-neck in large budding cells, which is the site of secretion (arrow). The same dual localization was seen with another dimeric PHOsh2 construct with only a single GFP, and much narrower definition of PHOsh2 (106 residues, pTL512, data not shown). B, cells expressing GFP-PHOsh2-dimer as in A, co-stained with the endocytic tracer FM4-64 (15 min uptake), which has entered late Golgi compartments. In the bottom panel, each image has been falsely colored: GFP in green, FM4-64 in magenta, with co-localization indicated in white. The very brightest punctae all showed good co-localization, as did a proportion of the less bright punctae (arrowhead). Using these settings, cells containing single markers showed complete separation of the fluorophores (data not shown). C, GFP-PHOSBP dimer as in A, but both PH domains of the dimer carry mutations in two basic residues implicated in PtdInsP binding (K307E/R309E, pTL513). The construct was entirely cytoplasmic, indicating loss of both of the localizations of wild-type PHOsh2.

co-workers (38). Indeed, of the 33 PH domains in the entire yeast genome, these two are among only seven that demonstrably bind PtdInsPs at all (38). Given a similar binding affinity for PtdInsPs, what determines the difference in targeting? This was mapped to the third residue of strand β7, which in PHOsh1 (tight binding) consists of RWHLKG, compared with RWRLKG in PHOsh2 (weak binding). The histidine is conserved in Osh1p homologues in humans (OSBP, ORP4, and ORP9), flies, worms, plants, and in other fungi (Candida albicans, Aspergillus nidulans but not Schizosaccharomyces pombe-serine, or Magnaporthe grisea and Neurospora crassa-threonine), whereas the arginine is unique to Osh2p in Saccharomyces. Different PH domain structures all indicate that this residue lies at the bottom of a shallow groove on a face of the domain adjacent to but not overlapping with the PtdInsP binding site (Fig. 2) (28, 29, 37). The finding that this histidine can specify Golgi localization identifies this side of PH domains as a site in this subfamily of PH domains that binds a second ligand. Given the location of the site away from the membrane, it would appear that the ligand is a protein, rather than a membrane lipid. Following our initial studies where we found that this second site depends on Arf1p in yeast (12), De Matteis and co-workers (21) have now demonstrated a direct interaction between both PHFAPP1 and PHOSBP with ARF1 in mammalian cells.

**FIG. 5. Different PtdIns 4-kinases synthesize the two pools of PtdIns(4)P.** All images are of the GFP-PHOsh2 dimer expressed (from pTL511) in the indicated mutant strains and the corresponding wild-type parental strains, grown to mid-log phase at the permissive temperature (25 °C) and shifted to a strongly non-permissive temperature (39 °C) for 15 min prior to imaging. A, inactivation of Pik1p completely inhibited the punctate localization of PHOsh2. B, targeting to both sites was unaffected by inactivation of Mss4p, indicating no requirement for PtdIns(4,5)Ps for localization. C, plasma membrane localization was lost selectively on inactivation of Stt4p, but not in the wild-type control. Plasma membrane targeting was also reduced in the stt4-4 mutant strain at a temperature permissive for growth (25 °C, data not shown).
predict that ARF1/Arf1p interacts with histidine 79 in PH\textsuperscript{Osh2} and PH\textsuperscript{OSBP}.

Although Golgi targeting can be achieved by combining two binding interactions, without a second site monomeric PH\textsuperscript{Osh2} does not target the Golgi. This reduced affinity for the Golgi may be crucial to targeting of full-length Osh2p, which has diverged in multiple ways from Osh1p (25). Because PH\textsuperscript{Osh2} interacts only with PtdInsPs, we have been able to develop a tool based on dimeric PH\textsuperscript{Osh2} that detects PtdIns(4)P and PtdIns(4,5)P\textsubscript{2} alone (Fig. 3). Binding to other PtdInsPs was shown to be irrelevant by the lack of a role for Vps34p. The dual specificity for PtdIns(4)P and PtdIns(4,5)P\textsubscript{2} (39). CERT was recently shown to transfer ceramide from punctate to plasma membrane targeting. Monomeric PH\textsuperscript{Osh2} showed enhanced targeting to the plasma membrane (compare with Fig. 1A). In comparison, PH\textsuperscript{Osh1}, PH\textsuperscript{OSBP}, and PH\textsuperscript{FAPP1} targeted punctae predominantly in \textit{Sac1} cells. In addition, these sequences showed minor targeting to the nuclear envelope (arrows) and cell periphery (arrowheads). PH\textsuperscript{CERT} was unique in targeting the ER (nuclear envelope and peripheral patches). This was dependent on binding to PtdInsPs, as it was abolished by mutating the critical basic residue in PH\textsuperscript{CERT} (Arg\textsuperscript{43}) to an acid (Glu).

FIG. 6. PH\textsuperscript{Osh2} and other Golgi-targeted PH domains reveal an altered distribution of PtdIns(4)P in cells lacking Sac1p. Images of GFP-tagged PH domains expressed in a \textit{Sac1} strain, grown to mid-log phase at 30 °C. A, GFP-PH\textsuperscript{Osh2} dimer; B, GFP-PH\textsuperscript{Osh2} monomer; C, GFP-PH\textsuperscript{Osh1}; D, GFP-PH\textsuperscript{OSBP}; E, GFP-PH\textsuperscript{FAPP1}; F, GFP-PH\textsuperscript{CERT}; and G, GFP-PH\textsuperscript{CERT}\textsuperscript{R43E} carrying the mutation R43E. The constructs used are indicated diagrammatically above each image (2-2, 1, O, F, C, C').

Although Golgi targeting can be achieved by combining two binding interactions, without a second site monomeric PH\textsuperscript{Osh2} does not target the Golgi. This reduced affinity for the Golgi may be crucial to targeting of full-length Osh2p, which has diverged in multiple ways from Osh1p (25). Because PH\textsuperscript{Osh2} interacts only with PtdInsPs, we have been able to develop a tool based on dimeric PH\textsuperscript{Osh2} that detects PtdIns(4)P and PtdIns(4,5)P\textsubscript{2} alone (Fig. 3). Binding to other PtdInsPs was shown to be irrelevant by the lack of a role for Vps34p. The dual localization of the GFP-PH\textsuperscript{Osh2} dimer in log-phase cells was consistent with previous studies of the two essential PtdIns 4-kinases (9), and their localization (10, 17). Whereas previous studies have shown that Pik1p produces PtdIns(4)P at the Golgi (12, 13), this is the first demonstration that Stt4p synthesizes PtdIns(4)P on the plasma membrane. Because the Stt4-dependent pools of PtdIns(4)P and PtdIns(4,5)P\textsubscript{2} are of roughly equivalent size (11), it is difficult to determine which lipid is being detected by the GFP-PH\textsuperscript{Osh2} dimer, even using cells in which Mss4p can be inactivated.

If the GFP-PH\textsuperscript{Osh2} dimer is indeed a probe for 4-phosphorylated PtdInsPs, its distribution should change in response to changes in PtdInsP metabolism. We used cells in which the SAC1 gene was disrupted, because in the absence of this PtdInsP phosphatase, cells accumulate up to 10 times the normal levels of PtdIns(4)P, whereas PtdIns(4,5)P\textsubscript{2} decreases by 50% (16, 32). Results with PH\textsuperscript{Osh2} in these cells showed that excess PtdIns(4)P accumulated mainly on the plasma membrane. However, other PH domains that bind PtdIns(4)P, such as those from Osh1p, OSBP, and FAPP1, remained largely localized to the Golgi in \textit{Sac1} cells, similar to the recent findings of Lemmon and coworkers (38). This is further evidence that PH domains that target the Golgi as monomers localize using a second receptor in addition to PtdInsP. Interestingly, PH\textsuperscript{CERT} behaved differently in cells carrying \textit{Sac1} compared with the other sequences tested in targeting the ER in a PtdInsP-dependent manner. This suggests that PH\textsuperscript{CERT} recognizes a second Golgi receptor that is relocated to the ER in a \textit{Sac1} strain. Because this receptor differs from that recognized by PH\textsuperscript{Osh1}, PH\textsuperscript{OSBP}, and PH\textsuperscript{FAPP1}, it may be that PH\textsuperscript{CERT} does not bind ARF1. ER relocalization of a Golgi protein in \textit{Sac1} cells has also been reported for the short yeast OSBP homologue Kes1p, which also binds PtdIns(4)P and PtdIns(4,5)P\textsubscript{2} (39). CERT was recently shown to transfer ceramide from punctate to plasma membrane targeting. Monomeric PH\textsuperscript{Osh2} showed enhanced targeting to the plasma membrane (compare with Fig. 1A). In addition, these sequences showed minor targeting to the nuclear envelope (arrows) and cell periphery (arrowheads). PH\textsuperscript{CERT} was unique in targeting the ER (nuclear envelope and peripheral patches). This was dependent on binding to PtdInsPs, as it was abolished by mutating the critical basic residue in PH\textsuperscript{CERT} (Arg\textsuperscript{43}) to an acid (Glu).

It is clear from all studies of PH domains targeted to Golgi membranes that they bind PtdIns(4)P. However, these PH domains can also show fairly indiscriminate binding to a broad range of PtdInsP (12, 24, 36, 38), i.e. there is no intrinsic specificity for PtdIns(4)P. This is sometimes overlooked (41) because different techniques for measuring protein-lipid interaction yield differing results. Binding protein to lipids immobilized on nitrocellulose (so-called “Fat Western”) originally showed that PH\textsuperscript{FAPP1} specifically binds PtdIns(4)P (20), however, this specificity was not seen with our lipid-probe technique. Although it is not yet known if other proteins that target PtdIns(4)P on the Golgi are equally promiscuous (22), it is tempting to speculate why such lack of specificity exists for PtdIns(4)P-binding proteins. PtdIns(4)P exists in multiple discrete pools, not only in yeast, but also in mammalian cells where multiple organelles can recruit PtdIns(4)Ps (4–8, 22, 42). Thus, proteins that target PtdIns(4)P can only achieve a narrow distribution among the multiple pools of PtdIns(4)P by combining this interaction with others. Therefore, there may be very little evolutionary disadvantage to binding the combination of PtdIns(4)P and PtdIns(4,5)P\textsubscript{2}, and it is possible that a truly PtdIns(4)P-specific binding protein may not exist.

In summary, PH\textsuperscript{Osh2} appears to detect two independent pools of 4-phosphorylated PtdInsPs without the bias introduced by a second site that is otherwise found in PH domains that bind PtdIns(4)P and PtdIns(4,5)P\textsubscript{2}. The potential use of the probe is complicated by its interaction with two lipids. Now that the microheterogeneity of all intracellular organelles has become evident (43–45), it will become increasingly important to study how multiple targeting signals integrate to specify the overall distribution of a protein.

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