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Targeting of Golgi-Specific Pleckstrin Homology Domains Involves Both PtdIns 4-Kinase-Dependent and -Independent Components

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Summary

Background: Phosphoinositides are required for the recruitment of many proteins to both the plasma membrane and the endosome; however, their role in protein targeting to other organelles is less clear. The pleckstrin homology (PH) domains of oxysterol binding protein (OSBP) and its relatives have been shown to bind to the Golgi apparatus in yeast and mammalian cells. Previous in vitro binding studies identified phosphatidylinositol (PtdIns) (4)P and PtdIns(4,5)P_2 as candidate ligands, but it is not known which is recognized in vivo and whether phosphoinositide specificity can account for Golgi-specific targeting.
Results: We have examined the distribution of GFP fusions to the PH domain of OSBP and to related PH domains in yeast strains carrying mutations in individual phosphoinositide kinases. We find that Golgi targeting requires the activity of the PtdIns 4-kinase Pik1p but not phosphorylation of PtdIns at the 3 or 5 positions and that a PH domain specific for PtdIns(4,5)P_2 is targeted exclusively to the plasma membrane. However, a mutant version of the OSBP PH domain that does not bind phosphoinositides in vitro still shows some targeting in vivo. This targeting is independent of Pik1p but dependent on the Golgi GTPase Arf1p.
Conclusions: Phosphorylation of PtdIns at the 4 position but not conversion to PtdIns(4,5)P_2 contributes to recruitment of PH domains to the Golgi apparatus. However, potential phosphoinositide ligands for these PH domains are not restricted to the Golgi, and the OSBP PH domain also recognizes a second determinant that is ARF dependent, indicating that organelle specificity reflects a combinatorial interaction.

Introduction

Phosphoinositides have been found to play key roles in the recruitment of specific proteins to membranes from the cytosol [1, 2]. These recruitment events are involved in signal transduction processes, cytoskeletal organization, and membrane trafficking. PtdIns can be phosphorylated by families of specific kinases at three of the five hydroxyl groups of its inositol headgroup (positions 3, 4, and 5) to produce seven different phosphoinositides. By restricting the action of the individual kinases to a subset of membranes and in some cases coupling their action to activation by signaling pathways the cell is able to use specific phosphoinositides to control the subcellular localization of particular proteins and alter this in response to external stimuli. Thus, PtdIns(3,4,5)P_3 is transiently induced at the plasma membrane in response to a number of external ligands, including insulin and growth factors, while PtdIns(4,5)P_2 and PtdIns(3)P are constitutively present on the plasma membrane and endosome, respectively [3–5].

Differential targeting of particular proteins in response to these different phosphoinositide distributions requires specific recognition of particular phosphoinositide species, and several domains have been identified that are able to carry out such recognition. The largest family are the pleckstrin homology (PH) domains which are found in a wide range of signal transduction and cytoskeletal proteins and bind to phosphoinositides with a range of binding affinities and specificities [6, 7]. Although many PH domains bind weakly or with broad specificity, a number show high affinity and specificity. Thus, the PH domain of phospholipase C δ1 (PLCδ1) binds with high affinity and specificity to PtdIns(4,5)P_2, while other PH domains show specificity for PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 and are found in proteins such as the kinases PDK1, Btk, and Akt that are recruited transiently to the plasma membrane in response to these second messengers [1, 8]. In addition to PH domains, further domains have been identified which bind to PtdIns(4,5)P_2, such as the ENTH domain, and it appears that a wide range of proteins use PtdIns(4,5)P_2 as a targeting determinant for the plasma membrane [9, 10]. In addition, the FYVE and PX domains found on a number of endosomal proteins have been shown to bind specifically to PtdIns(3)P [11, 12].

This raises the question of whether phosphoinositides could play a role in targeting to other organelles, such as the Golgi [13]. We have recently examined the PH domain of oxysterol binding protein (OSBP), a protein of unknown function which was identified as binding to oxysterols, oxygenated derivatives of cholesterol which are potent regulators of a number of aspects of cholesterol metabolism [14, 15]. OSBP translocates to the Golgi in the presence of oxysterols, and the PH domain of OSBP (PH^{OSBP}) is sufficient to confer specific targeting to Golgi membranes [16, 17]. OSBP is the founder member of a large protein family found in all eukaryotes [18], and Osh1p, a homolog of OSBP from the yeast *S. cerevisiae*, is also targeted to the Golgi in yeast [19].

This raises the question of what determinant is recognized by these Golgi-specific PH domains. PH^{OSBP} was found to bind to PtdIns(4)P and to PtdIns(4,5)P_2 in vitro [17], but it is not known whether either of these lipids are involved in the targeting of PH^{OSBP} in vivo, and, if so, how they could contribute to Golgi specificity as, at least, PtdIns(4,5)P_2 is known to be present at the plasma membrane. To address this question, we have examined

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the targeting of Golgi-specific PH domains in yeast strains in which kinases involved in phosphoinositide synthesis can be inactivated and compared this to the targeting of a PH domain with a known specificity for PtdIns(4,5)P_2. We find that PH^{OSBP} and a number of related PH domains from mammalian proteins are targeted to the Golgi in a manner dependent in part on PtdIns(4)P production on the Golgi. In contrast, the PtdIns(4,5)P_2-specific PH domain of PLCδ1 is targeted to the plasma membrane. Mutation of conserved basic residues in the putative binding pocket of PH^{OSBP} abolishes PtdIns binding in vitro but does not completely abolish targeting in vivo. This residual targeting appears independent of PtdIns(4)P but is lost upon inactivation of the Golgi-specific GTPase Arf1p. Taken together these results indicate that yeast Golgi membranes contain predominantly PtdIns(4)P rather than PtdIns(4,5)P_2, with PtdIns(4)P contributing to Golgi targeting of the OSBP PH domain in vivo. However, the intracellular distribution of these Golgi-specific PH domains is apparently determined by more than their lipid binding specificity, with a second, ARF-dependent factor also contributing to recruitment.

Results

Targeting of PH^{OSBP} and Related PH Domains in Yeast

The targeting of the PH domain of OSBP to the Golgi apparatus appears to be well conserved in evolution, as the PH domain of a yeast OSBP homolog, Osh1p, targets to the Golgi apparatus in mammalian cells as well as in yeast [17, 20]. Likewise, when the PH domain of human OSBP was expressed in yeast as a fusion protein to GFP it showed, as expected, the punctate distribution characteristic of the yeast Golgi, with no discernible accumulation on the plasma membrane (Figure 1B). GFP-PH^{OSBP} remained localized following fixation and showed substantial colocalization with the TGN marker Tlg1p (data not shown), similar to the colocalization of Tlg1p and a construct containing PH^{Osh1p} seen previously [20]. Recently, several proteins have been identified with PH domains that are related to that of OSBP, although they lack oxysterol binding domains and show no other similarity to OSBP. These include Goodpasture antigen binding protein (GPBP), a human protein that has homologs in invertebrates but not yeast [21], and PtdIns 4-phosphate adaptor protein-1 (FAPP1), a protein so far found only in mammals [22]. The functions of these proteins are unknown, but they suggest the existence of a wider group of PH domains that could share the properties of PH^{OSBP} (Figure 1A). We initially investigated whether the similarity in the sequences of these PH domains is reflected in similar intracellular targeting. First, we expressed GFP fusions with PH^{GPBP} and PH^{FAPP1} in yeast, and both these constructs also localized to punctate structures and did not appear to be on the plasma membrane (Figure 1B). In addition, when GFP-PH^{GPBP} and GFP-PH^{FAPP1} were expressed in COS cells, they localized to a juxta-nuclear reticulum, which colocalized with the Golgi marker TGN46 (Figure 1C), although in both COS cells and yeast the FAPP1 PH domain consistently showed a larger cytosolic fraction than the other two, suggesting that it recognized the Golgi with a lower affinity. Together, these results indicate that the properties of the OSBP PH domain are not only well conserved in evolution but are also shared with closely related PH domains from a wider group of proteins.

Efficient Golgi Targeting Requires PtdIns(4)P Production In Vivo

In our initial characterization of OSBP PH domain, we found that it bound with similar affinity to PtdIns(4)P and PtdIns(4,5)P_2. Both of the these phosphoinositides are found in yeast cells, along with two others, PtdIns(3)P and PtdIns(3,5)P_2 (Figure 2A). To examine which of these molecules contributed to the Golgi targeting in vivo, we made use of mutations in the kinases responsible for their synthesis. Two of the four phosphoinositides are not required for viability, PtdIns(3)P and PtdIns(3,5)P_2, and their synthesis is dependent upon the Vps34p PtdIns-3 kinase. Deletion of the *VPS34* gene had no effect on the punctate localization of GFP-PH^{OSBP} or of GFP-PH^{GPBP} (Figure 2B and data not shown), indicating that PtdIns(3)P and PtdIns(3,5)P_2 are not required for recruitment of these PH domains to membranes. Synthesis of the other phosphoinositides is essential, and so their roles were investigated with temperature-sensitive alleles of the relevant kinases.

Given the in vivo localization of these PH domains to the Golgi, we first investigated whether localization is mediated by Pik1p, a PtdIns 4-kinase found on late Golgi membranes [23–25]. When GFP-PH^{OSBP} chimera was expressed in yeast with a temperature-sensitive allele of *pik1*, incubation of the cells at a nonpermissive temperature for 15 min caused substantial, although not complete, delocalization of the reporter protein, while there was no change in the wild-type *PIK1* control cells (Figure 2B). The same inactivation of Pik1p also led to considerable delocalization of PH^{GPBP} and PH^{FAPP1} (Figure 2B and data not shown). These results indicate that PH^{OSBP} and related PH domains require functional Pik1p for normal membrane recruitment. Yeast contain a second essential PtdIns 4-kinase, the product of the *STT4* gene. This kinase appears to be responsible for the synthesis of a pool of PtdIns(4)P at the plasma membrane [26], and indeed the localization of the PH domains was unaffected in a temperature-sensitive allele at the nonpermissive temperature (data not shown).

The PtdIns(4)P made by Pik1p could serve in PH domain recruitment either by playing a direct role itself or by providing a substrate for synthesis of PtdIns(4,5)P_2 that then functions in the recruitment to Golgi membranes. To test this latter possibility, we next examined the effect of a temperature-sensitive allele of *MSS4*, the only known PtdIns(4)P 5-kinase in yeast [27, 28] (Figure 2A). Inactivation of Mss4p had no effect on the localization of PH^{OSBP} (Figure 2B), even though these conditions delocalize the PtdIns(4,5)P_2-specific PH domain from PLCδ1 (see below). Similarly, PH^{FAPP1} and PH^{GPBP} also showed no delocalization in a *mss4-2^{ts}* strain (data not shown). Taken together, these results suggest that targeting of PH^{OSBP} and related PH domains to Golgi membranes requires PtdIns(4)P and not PtdIns(4,5)P_2 derived from it or any of the 3-phosphorylated phosphoinositides.

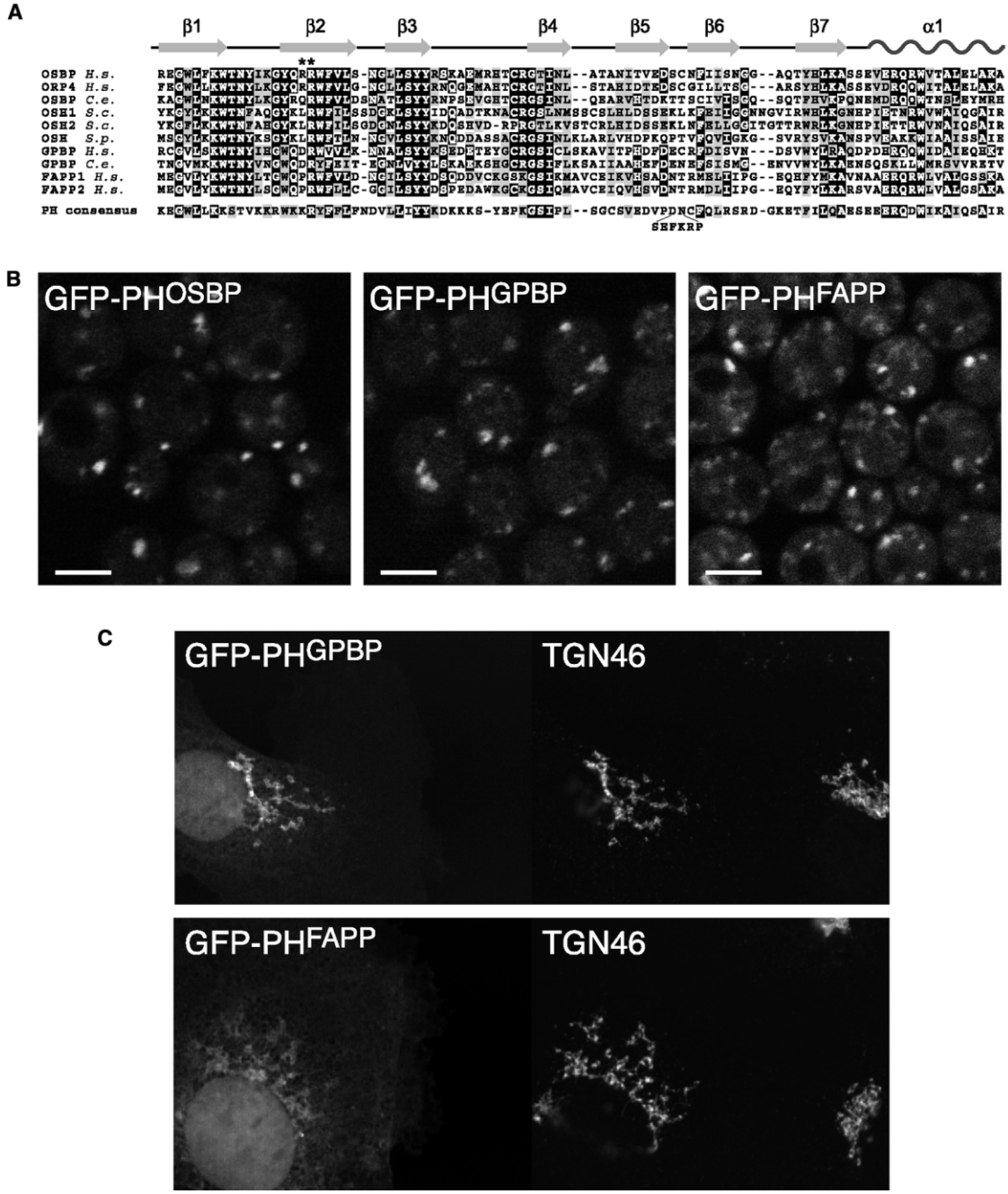

Figure 1. The PH Domain of OSBP Is a Member of a Family of Related PH Domains which Target to the Golgi in Yeast and Mammalian Cells

(A) Alignment of PH^{OSBP} and closely related PH domains. Residues that are identical (black) or related (gray) in five or more of the ten sequences are shaded, indicating a high degree of homology (in 67/96 positions) despite the divergent origin of the sequences, in terms both of protein family and of species (*H.s., H. sapiens; C.e, C. elegans; S.p., S. pombe; S.c., S. cerevisiae*). The PH consensus is that from PFAM based on 163 PH domains (pfam00169, http://www.ncbi.nlm.nih.gov/Structure/). The structure of known PH domains is shown above the alignment—seven β strands, followed by an α helix. Asterisks indicate the two arginines in OSBP that were mutated to glutamate in this paper.

(B) Confocal images of live wild-type yeast cells expressing GFP fusions to PH domains from OSBP, FAPP1, and GPBP (strain RS453B, carrying plasmids pTL332-334, respectively).

(C) GFP-PH^{GPBP} and GFP-PH^{FAPP1} colocalize with a Golgi marker in COS-1 cells. Confocal micrographs of COS cells fixed 40 hr after transient transfection (plasmids pBGPk or pBGPm), permeabilized, and the Golgi marker TGN46 localized by immunofluorescence. Scale bars, 3 μm.

$PH^{PLC\delta1}$ Identifies a Pool of PtdIns(4,5)P_2 on the Yeast Plasma Membrane

Previous studies have found that the PH domain of OSBP can bind to both PtdIns(4)P and PtdIns(4,5)P_2 in vitro ([17, 29] and see below). Thus, PtdIns(4,5)P_2 might have been expected to play a role in the targeting of the OSBP PH domain in vivo. The PH domain of PLCδ1 has been shown to bind specifically to PtdIns(4,5)P_2 and not to PtdIns(4)P [30], and chimeras between GFP and the PH domain have been successfully used to monitor

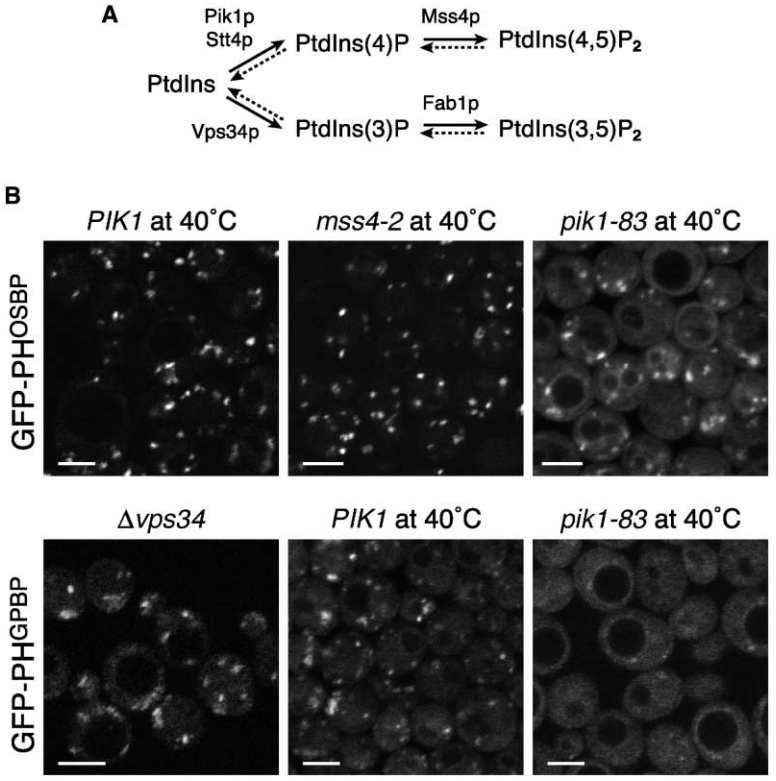

Figure 2. Requirement for Pik1p for the Targeting of Golgi Localized PH Domains

(A) The pathways of phosphoinositide synthesis in *S. cerevisiae* and the lipid kinases known to be required.

(B) Confocal micrographs of live yeast of the indicated genotypes, expressing GFP-PH^{OSBP} or GFP-PH^{GPBP}. Cells (*PIK1*, YES32; *mss4-2^{ts}*, SD102; *pik1-83*, YES102; Δ*vps34* PHY102) transformed with plasmids pTL333 or pTL332, respectively, were grown to log-phase at 25°C and incubated at 40°C for 15 min prior to microscopy, except for Δ*vps34*, which was grown at 30°C throughout. In the *pik1-83* strain, not only is there an increase of cytoplasmic staining, but there is some relocalization of GFP-PH^{OSBP} to both the plasma membrane and the vacuolar membrane. The reasons for this are unclear but may reflect a weak affinity for membranes that is revealed when the specific ligand is unavailable. At 7 min after temperature shift, delocalization is less extensive (data not shown). Temperature shift in wild-type cells causes slight delocalization of GFP-PH^{GPBP} and has no significant effect on GFP-PH^{OSBP}. Incubation at 39°C affects both PH domains to a lesser extent and at 38°C or 37°C is without effect (data not shown). This requirement for temperatures above that restrictive for cell growth may be related to the partial thermoresistance of *pik1* alleles [39]. Scale bars, 3 μm.

PtdIns(4,5)P_2 in living mammalian cells [4, 5]. Thus, to investigate the possibility that PtdIns(4,5)P_2 could be involved in targeting to the Golgi, we constructed a fusion protein containing GFP and the PH domain (Figure 3A), and when this chimera, GFP-PH$^{PLC\delta1}$, was expressed in yeast, it showed some concentration on the limiting membrane of yeast cells but also substantial diffuse cytoplasmic fluorescence (Figure 3B). In order to increase the proportion of the construct binding to target membranes, we increased the avidity of the interaction by constructing a chimera containing two copies of the PH$^{PLC\delta1}$ domain (Figure 3A). This GFP-PH$^{PLC\delta1}$-dimer construct showed strong plasma membrane localization and almost no localized internal fluorescence (Figure 3B). To confirm that the plasma membrane targeting of PH$^{PLC\delta1}$-dimer was dependent on PtdIns(4,5)P_2, the GFP fusion was expressed in yeast containing a temperature-sensitive allele of *MSS4*, the only kinase in yeast that synthesizes PtdIns(4,5)P_2. Plasma membrane targeting was observed in the mutant cells at the permissive temperature (data not shown), but after 30 min incubation at 37°C the construct had predominantly delocalized from the plasma membrane, with a concomitant increase in diffuse background, a change not seen in cells containing wild-type *MSS4* (Figure 3C). These results indicate that PH$^{PLC\delta1}$-dimer recognizes PtdIns(4,5)P_2 in yeast cells and suggest that the majority of this ligand is on the plasma membrane rather than on the Golgi.

Mutation of Conserved Basic Residues in OSBP Blocks PtdIns(4)P Binding In Vitro

If recognition of PtdIns(4)P is involved in targeting the OSBP PH domain to the Golgi, then a mutant that has lost this binding in vitro should also be unable to target in vivo. The determination of the structure of a number of PH domains has revealed that ligand binding is meditated by residues, usually basic, in the β strands and loops forming a binding pocket [2, 31]. Residues in strands 1 and 2 play a role in all PH domains so far examined, and the OSBP PH domain has two arginine residues in strand 2, one of which is conserved in OSBP relatives and also in many other PH domains, and, where structural information is available, it has been found to interact with phosphoinositide phosphates.

Thus, to examine if the ability of the OSBP PH domain to target the Golgi was dependent its ability to bind to phosphoinositides, a mutant version of the PH domain was used in which the two adjacent arginines were mutated to glutamate [R(107)E;R(108)E (RR > EE), Figure 1A]. Initially, this mutant was purified from *E. coli* as a GFP fusion protein, and its ability to bind liposomes containing phosphoinositides was compared to other PH domains expressed as GFP fusion proteins. Figure 4 shows that the wild-type OSBP PH domain bound to liposomes containing PtdIns(4)P or PtdIns(4,5)P_2, as expected [17, 29], while the mutant form showed no detectable binding, even with high levels of phosphoinositide. A GFP fusion to the PH domain from PLCδ1 bound PtdIns(4,5)P_2 but not PtdIns(4)P as previously reported, and fusions with the PH domains from GPBP and FAPP1 showed a similar binding to those of OSBP, although the affinities of the latter were somewhat lower (legend to Figure 4). As noted above, the FAPP1 PH domain showed a greater cytosolic pool when expressed in both yeast and COS cells, suggesting that its in vivo interaction with the Golgi may also be weaker.

When the mutant form of the OSBP PH domain was

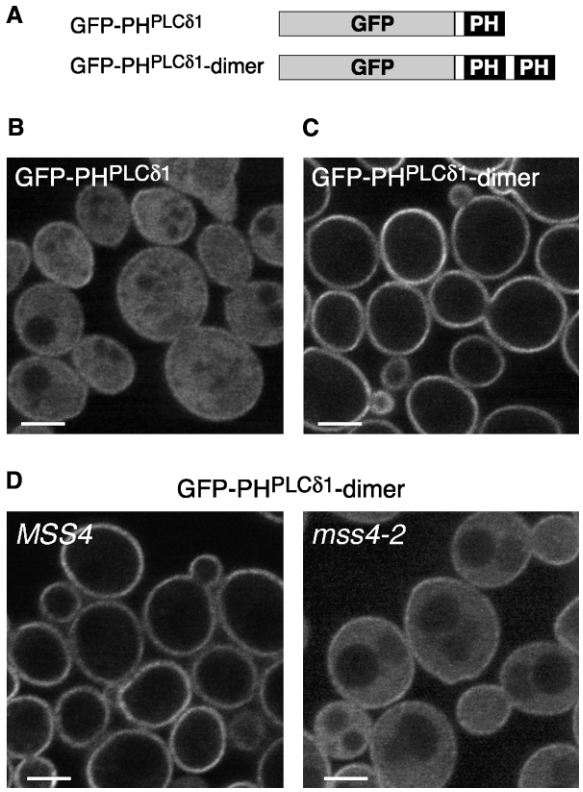

Figure 3. Targeting of PH$^{PLC\delta1}$ to the Yeast Plasma Membrane Is Dependent on PtdIns(4,5)P_2

(A) Structure of the GFP fusion proteins used, comprising GFP and one or two copies of the PLCδ1 PH domain.

(B) Confocal images of live wild-type cells (RS453B) expressing GFP-PH$^{PLC\delta1}$ or GFP-PH$^{PLC\delta1}$-dimer (plasmids pTL335 or pTL336, respectively).

(C) *MSS4* is required for PH$^{PLC\delta1}$-dimer localization. Confocal images of live yeast expressing GFP-PH$^{PLC\delta1}$-dimer (from plasmid pTL336) either harboring *mss4-2^{ts}* (SD102) or *MSS4* wild-type controls (SD100). Cells were grown at 20°C and shifted to 37°C for 30 min prior to confocal microscopy. Scale bars, 3 μm.

expressed in live yeast, it was mostly in a diffuse cytosolic distribution (Figure 5A). However, some punctate structures were still visible, which were never seen with untagged GFP, suggesting that the mutant still retained some capacity to bind membranes. To investigate this further, a dimeric form of the mutant PH domain was expressed in yeast, and this now gave clear punctate localization with little diffuse fluorescence (Figure 5A). Some of this material remained localized following fixation and showed overlap with the late Golgi SNARE Tlg1p (Figure 5B). Moreover, when this dimeric version of the mutant PH domain was expressed in mammalian cells, it was clearly able to target to the Golgi apparatus, although the monomeric mutant had only shown faint Golgi staining in some cells [17]. We had previously found that overexpression of the wild-type OSBP PH domain in COS cells causes a disruption of the *trans* side of the Golgi, and the same was true for the dimeric form of the mutant protein, with displacement of the TGN coat protein AP1 at higher levels of expression (Figure 5C).

Taken together, these results indicate that a form of the OSBP PH domain that does not bind to PtdIns(4)P or PtdIns(4,5)P2 is no longer as efficiently targeted to the Golgi apparatus as the wild-type domain, and this residual targeting suggests that a phosphoinositide-independent factor is involved.

PtdIns(4)P-Independent Targeting of the OSBP PH domain Requires the ARF GTPase

To obtain further evidence for the possibility that an aspect of OSBP targeting is phosphoinositide independent, the dimer mutant was expressed in the temperature-sensitive allele of *PIK1*. At the nonpermissive temperature, the dimer was not displaced into the cytoplasm (Figure 6A), consistent with this targeting being independent of PtdIns(4)P and indicating that membrane targeting did not reflect some residual binding to PtdIns(4)P in vivo that was undetectable in vitro.

The recruitment of a number of proteins to the Golgi apparatus is known to be dependent on members of the ADP-ribosylation factor (ARF) family of small GTPases. *S. cerevisiae* contains an essential pair of Golgi ARFs encoded by the genes *ARF1* and *ARF2*, and, when the mutant dimer was expressed in a strain in which the only Golgi ARF was a temperature-sensitive allele of *ARF1* (*arf1-11*), then it was displaced from the Golgi at the nonpermissive temperature (Figure 6A). Interestingly, the wild-type monomeric OSBP PH domain was also efficiently delocalized in the *arf1-11* strain at the nonpermissive temperature, suggesting that both the PtdIns(4)-independent but also PtdIns(4)-dependent recognition of the Golgi is dependent on Arf1p activity (Figure 6B). It is has been reported that *arf1* mutants have reduced PtdIns(4)P levels and, in mammalian cells, that one of the proteins recruited to the Golgi by ARF is the PIK4β PtdIns 4-kinase itself [32, 33].

The Golgi is known to remain intact in the *arf1-11* strain at the nonpermissive temperature [34], although the shape of the Golgi changes to be larger and ring-like, a phenomenon observed in a number of mutants that affect Arf activity [34–36]. Interestingly, similar ring-like structures were occasionally observed in cells expressing high levels of GFP fusions to the PH domains of OSBP and its relatives. To examine this apparent dominant-negative effective further, these PH domains were overexpressed using an inducible *GAL1* promoter. Figure 6C shows that under such circumstances rings of staining are visible in many cells, and examination of multiple focal planes indicates that, as for the structures seen in the *arf* mutants, they appear to be hollow spheres (Figure 6D).

Together these results indicate that targeting of the PH domain of OSBP to Golgi membranes involves recognition of a determinant in addition to PtdIns(4)P and that generation of this second determinant requires the activity of the ARF GTPase. Moreover, overexpression of the PH domain appears to phenocopy the morphological effects on the Golgi of reducing Arf1p activity, consistent with the PH domain titrating out a determinant that is required for other Arf1p-mediated Golgi processes.

Discussion

Recognition of distinct phosphoinositide species by PH domains has been shown to play a major role in the

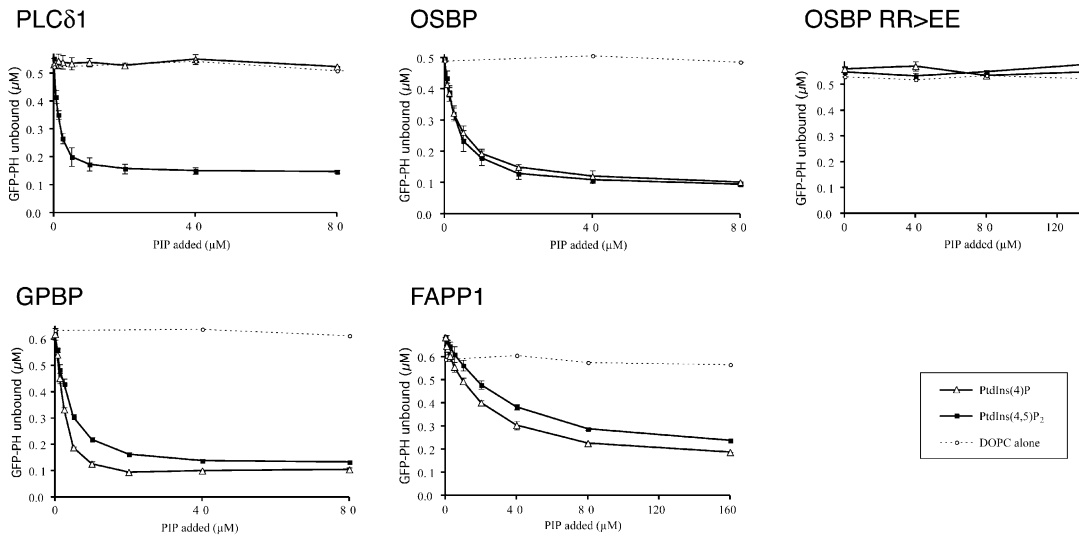

Figure 4. Binding of PH Domains to Phosphoinositide-Containing Liposomes

Binding of fusion proteins comprising GFP and the indicated PH domains to increasing amounts of liposomes containing the indicated phosphoinositide or equivalent amount of liposomes with no phosphoinositide (DOPC alone). Fusion proteins expressed in bacteria were incubated with raffinose-loaded liposomes containing 2.5% phosphoinositide, the liposomes pelleted by centrifugation and binding quantified by measuring the amount of GFP that remained in the supernatant (GFP-PH unbound). Binding was assayed in triplicate with standard deviations indicated. Kds were for PtdIns(4,5)P_2: OSBP, 3.4 μM ± 1.0; GPBP, 4.0 μM ± 0.7; FAPP1, 33.2 μM ± 7.9; PLCδ1, 1.1 μM ± 0.4; and for PtdIns(4)P: OSBP, 3.5 μM ± 0.7; GPBP, 2.0 μM ± 0.1; FAPP1, 18.6 μM ± 7.3.

regulated and constitutive recruitment of proteins to the plasma membrane [1, 7]. We had previously found that the PH domain of the Golgi peripheral membrane protein OSBP was sufficient to target a reporter protein to the *trans*-Golgi. In vitro this PH domain bound to both PtdIns(4)P and PtdIns(4,5)P_2, and recruitment of the PH domain to Golgi membranes in vitro could be blocked by phospholipase cleavage of phosphoinositides [17]. However, these results raised the question of which phosphoinositide was being recognized on the Golgi in vivo, especially because a number of PH domains and other proteins that specifically recognize PtdIns(4,5)P_2 are targeted to the plasma membrane and not to the Golgi in mammalian cells [4, 5, 10, 37]. In this paper, we have been able to provide some resolution of these questions. First, investigation of the targeting of the PH domain of OSBP in yeast strains with mutations in PtdIns kinases showed that phosphorylation of PtdIns at either the 3 or 5 positions was not required for Golgi targeting, but inactivation of the PtdIns 4-kinase Pik1p did affect targeting. Moreover, a PH domain specific for PtdIns(4,5)P_2 is predominantly targeted to the plasma membrane of yeast cells.

Our results suggest that Golgi-specific PH domains recognize PtdIns(4)P in vivo rather than PtdIns(4,5)P_2. A role for PtdIns(4)P in Golgi function is consistent both with its apparent role in membrane traffic from the Golgi apparatus [25, 32, 38] and with the previously reported Golgi localizations of PI4Kβ in mammals and Pik1p in yeast [25, 33, 39]. Although the PH domain of OSBP can bind to both PtdIns(4)P and PtdIns(4,5)P_2, there is no need to evoke the presence of PtdIns(4,5)P_2 on the Golgi to explain its targeting. Indeed, the plasma membrane targeting of a PtdIns(4,5)P_2-specific PH domain in both yeast and mammalian cells [4, 5], along with the plasma membrane localization of the Mss4p PtdIns 5-kinase [28], raises the possibility that there is little PtdIns(4,5)P_2 present on the Golgi in vivo. In mammalian cells, a large number of proteins that are specifically recruited to the plasma membrane recognize PtdIns(4,5)P_2, and it may be that PtdIns(4)P is used by the cell as a targeting determinant on the Golgi, to allow the organelle to be free of PtdIns(4,5)P_2 so that recognition of this latter phosphoinositide can be sufficient to confer a plasma membrane location.

However, phosphoinositide binding alone cannot account for the Golgi targeting of Golgi-specific PH domains. The PH domain of OSBP binds to PtdIns(4,5)P_2 in vitro [17, 29], and this seems to be also true for the PH domains of GPBP and FAPP1, and yet these PH domains do not bind to the plasma membrane pool of PtdIns(4,5)P_2. A previous study of the PH domain of FAPP1 has suggested a preference for PtdIns(4)P over PtdIns(4,5)P_2, but this may possibly reflect the use of a less physiological substrate, nitrocellulose strips rather than lipid bilayers, for presentation of the phosphoinositides [22]. Moreover, it is unlikely that PtdIns(4)P itself is solely restricted to Golgi membranes, as not only does it seem probable that there is a pool of PtdIns(4)P at the plasma membrane to provide a substrate for PtdIns(4,5)P_2 synthesis [28], but also the two essential PtdIns 4-kinases in yeast, *PIK1* and *STT4*, have distinct phenotypes and cannot substitute for each other even when overexpressed [23, 24, 32, 40]. A novel PtdIns 4-kinase has recently been identified in mammalian cells, which has a yeast homolog, the product of a nonessential gene of unknown function (*LSB6*, [41]). No activity has been reported for this protein, and deletion

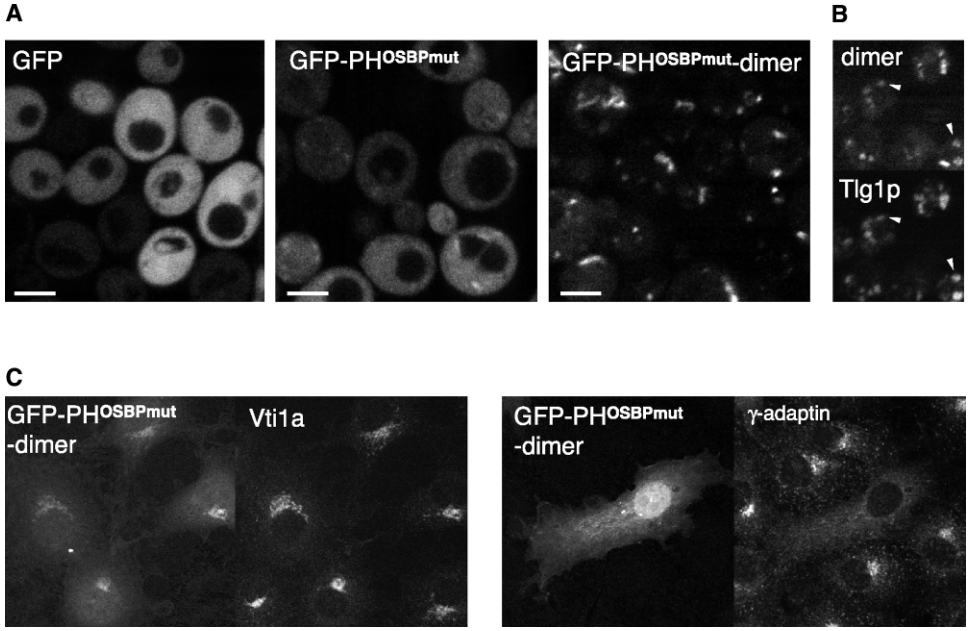

Figure 5. Targeting of a Mutant Form of the OSBP PH Domain to the Golgi in Yeast and Mammalian Cells

(A) Confocal images of live yeast expressing GFP either alone or as a fusion to one or two copies of the RR→EE mutant of the OSBP PH domain (plasmids pTL350-352, respectively). The structure of the monomeric or dimeric chimeras was as in Figure 3A.

(B) Immunofluorescent micrograph of yeast expressing the GFP-PH^{OSBPmut}-dimer, fixed and labeled with antibodies to the tSNARE Tlg1p. The GFP chimera and the late Golgi tSNARE show substantial colocalization, with a few structures unique to one or the other protein. Such a lack of complete colocalization with Tlg1p is often observed with Golgi markers in yeast [52].

(C) Immunofluorescent micrographs of COS cells expressing GFP-PH^{OSBPmut}-dimer and labeled with antibodies to the Golgi SNARE Vti1a or to the γ-adaptin component of the *trans*-Golgi AP1 adaptor complex. AP1 is displaced from the Golgi at high levels of expression of the chimera. Scale bars, 3 μm.

of the gene had no effect on the targeting of the OSBP PH domain (T.P.L., unpublished data). The notion that there is a second, PtdIns(4)P-independent factor that increases the specificity of Golgi targeting is supported by our observation that a mutant version of the PH domain of OSBP, which does not bind phosphoinositides, shows some residual Golgi targeting that becomes clearly apparent upon dimerization and is dependent on Arf1p but not Pik1p activity.

Taken together, these results suggest that Golgi membranes are recognized by PH domains via interaction with PtdIns(4)P but not PtdIns(4,5)P_2 and with a second factor whose presence requires the activity of Arf1p. Such a combinatorial interaction with a single PH domain would be analogous to the proposal that the PH domain of β-adrenergic receptor kinase binds both phosphoinositide and G protein β/γ subunits [42]. ARF proteins are required to recruit a number of proteins to Golgi membranes [43, 44], and so the PH domain could bind directly to one of these proteins or to a lipid generated by one of them. Indeed, it has been shown in mammalian cells that the Golgi targeting of a PtdIns 4-kinase is dependent on ARF1 [33], and, in yeast, a genetic interaction has been observed between *PIK1* and *ARF1*, which could provide an explanation for the complete loss of targeting of the wild-type OSBP PH domain when Arf1p is inactivated. The OSBP PH domain also recognizes PtdIns(4,5)P_2 in vitro, and yet it does not target to the plasma membrane, unlike the PtdIns(4,5)P_2-specific PH domain from PLCδ1. This seems likely to reflect the latter PH domain having a 4- to 5-fold higher affinity for PtdIns(4,5)P_2, as even with this higher affinity the plasma membrane targeting of monomeric GFP-PH$^{PLC\delta1}$ is only just detectable in vivo, presumably reflecting binding being mediated solely by interaction with PtdIns(4,5)P_2, rather than the combinatorial binding proposed for the OSBP PH domain which shows clear targeting even for the monomeric construct.

If OSBP recognizes a PtdIns(4)P-containing determinant specific to Golgi membranes, this raises the possibility that the intracellular distribution of other proteins is also dependent on this determinant. In our initial characterization of PH^{OSBP}, we found that overexpression resulted in fragmentation of the *trans* side of the mammalian Golgi [17], and in this paper we find that overexpression of the PH domain in yeast alters Golgi morphology, suggesting that it is competing with the targeting of other Golgi proteins. The Golgi-recognizing PH domains examined in this paper are found not only in OSBP and its relatives, but also in GPBP and FAPP1, members of two further protein families. The functions of these proteins are unknown, but both GPBP and a FAPP1 relative, FAPP2, contain additional domains related to known lipid binding proteins (StAR and glycolipid transfer protein, respectively [45, 46]), suggesting that all three families of proteins could be involved in the metabolism or sorting of lipids in the Golgi. However, of these three families, only OSBP has homologs in yeast, and deletion of the three yeast Osh genes containing PH domains does not affect viability or Golgi morphology

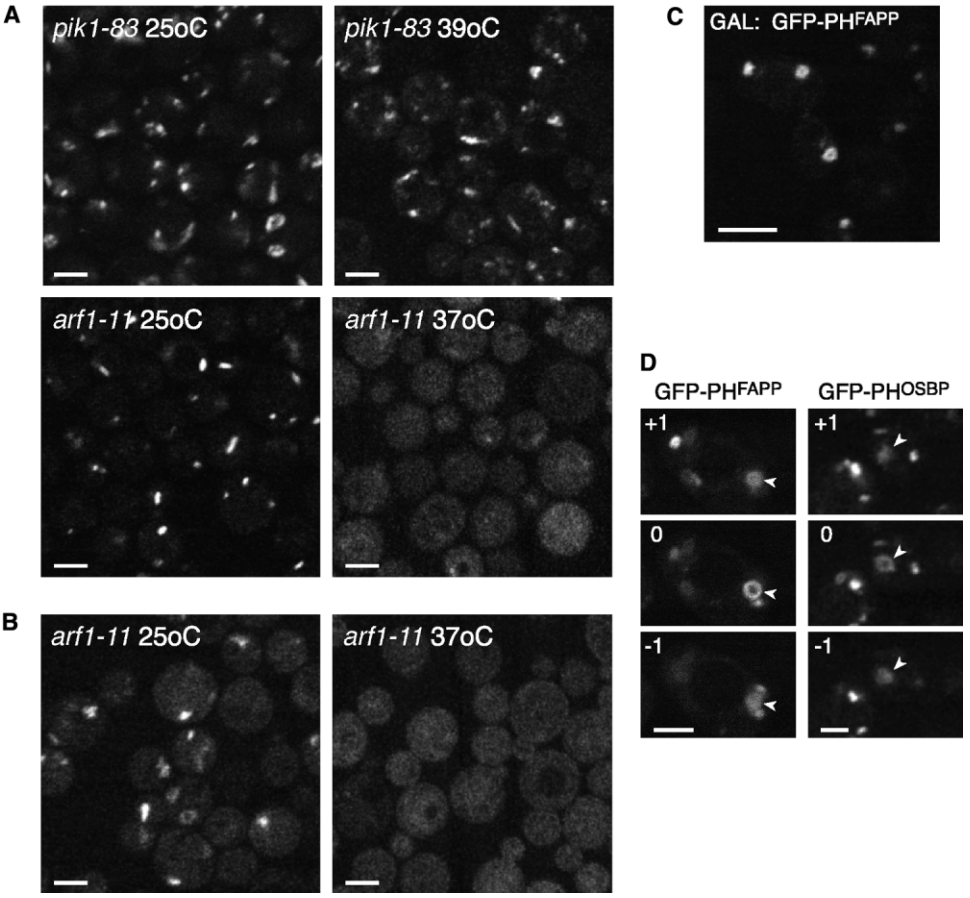

Figure 6. Targeting of OSBP PH Domain to the Yeast Golgi Is Dependent upon Activity of the Arf1p GTPase

(A) Confocal images of live yeast expressing GFP-PH^{OSBPmut}-dimer in the *pik1* or *arf1* temperature-sensitive strains (*pik1-83* as Figure 2; *arf1-11*, strain NYY11-2) after growth at 25°C and with or without elevation to the indicated temperature for 15 min.

(B) Confocal images of live yeast expressing GFP-PH^{OSBP} (wild-type, monomeric) in the *arf1-11* temperature-sensitive strain as in (A).

(C) Confocal images of live yeast expressing GFP-PH^{FAPP1} from a CEN plasmid under the control of the GAL1 promoter (pTL364) and photographed 90 min after induction in galactose.

(D) Stacks of three adjacent optical sections (0.5 μm apart) through live yeast expressing either GFP-PH^{FAPP1} or GFP-PH^{OSBP} as in (C). The ring-like structures seen in slice 0 have a top and bottom, indicating that they are spherical. Scale bars, 3 μm (A and B) or 2 μm (C and D).

[20, 47], implying that recruitment of proteins containing this set of PH domains cannot account for the requirement of Pik1p for normal secretion in yeast. This suggests that further proteins are recruited to the Golgi in a manner dependent upon the determinants recognized by the Golgi PH domains. There are a large number of peripheral membrane proteins involved in membrane trafficking processes in the Golgi, including coat proteins, coiled-coil proteins, and GEFs and GAPs for several small GTPases, all of which need to be recruited accurately. Use of multiple targeting determinants including PtdIns(4)P and Arf1p could provide a means to recruit such proteins in a manner that is both Golgi-specific but can also be regulated in response to the cell's changing needs for the level of protein and lipid traffic through the secretory pathway.

Experimental Procedures

Plasmid Construction

All constructs were expressed in yeast from plasmids based on the integrative *URA3* vector pRS406. The PH domains used were as follows (ORF names and accession numbers given in brackets), with starting and ending residues shown using single-letter amino acid code: OSBP (P22059) 87S→185K; GPBP (NP005704) 1M→122G; FAPP1 (AAG15199) 1M→98T; PLCδ1 (P10688) 1M→175Q. Plasmids expressing PH^{OSBP}, PH^{GPBP}, and PH^{FAPP1} in yeast as GFP fusions contain the constitutive portion of the *PHO5* promoter followed by GFP, a linker with the Myc epitope (GSMEQKLISEEDLRS), then the PH domain (pTL332-334, respectively). The plasmid expressing GFP-PH$^{PLC\delta1}$ (pTL335) has no Myc epitope, and the plasmid expressing GFP-PH$^{PLC\delta1}$-dimer (pTL336) contains a second copy of the PH domain adjacent to the first, separated by a short linker (GAGARS). pTL364 contains the same GFP-PH^{FAPP1} fusion protein as pTL334 but with the promoter replaced for that of *GAL1*. Mutated PH^{OSBP} (R107→E R108→E, pTL351) was cloned by Quikchange (Stratagene) into a plasmid similar to pTL332, except that the *TPI1* promoter is used. Dimeric mutant PH^{OSBP} (pTL352) has a second copy of the Myc-PH cassette inserted adjacent to the first, separated by a short linker (GAGAGS). pTL350 expresses GFP alone from the same promoter in pRS406. For expression in COS-1 cells, PH^{GPBP}, PH^{FAPP1}, and PH^{OSBP}(RR→EE)-dimeric sequences were substituted for PH^{OSBP} in pBGPa (a vector containing the CMV immediate early gene promoter, GFP, and the Myc epitope followed by the PH domain [17]) to make pBGPk, pBGPm, and pBGPn, respectively. For expression of His$_6$-tagged fluorescent proteins in bacteria, open reading frames

as outlined above, including GFP, were cloned into pTrcHisA (Invitrogen) as described previously [17].

Yeast Strains

For localization studies, plasmids were transformed into wild-type yeast strain RS453B [20] or strains harboring temperature-sensitive alleles (together with their wild-type controls) as follows. For *MSS4*: SD100 [*MAT***a** *leu2–3,112 ura3–52 trp1 his4 rme1 HML*α *mss4::HIS3MX6* carrying YCplac111(*CEN LEU2*)::*MSS4*] and *mss4-2*^{ts}: SD102 [SD100, but carrying YCplac111(*CEN LEU2*)::*mss4-2*] [27]. For *PIK1*: YES32 (*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*1 ura3-52 PIK1::TRP1*) and *pik1-83*^{ts}: YES102 (YES32 *pik1-83::TRP1*) [48]. For *STT4:* SEY6210 (*MAT*α *leu2-3,112 ura3-52 his3-*Δ*200 trp1-*Δ*901 lys2-801 suc2-*Δ*9)* and *stt4-4*^{ts}: AAY102 (SEY6210 *stt4::HIS3* carrying pRS415(*CEN LEU2*)::*stt4-4*) [32]. Δ*vps34* is PHY102 (SEY6210 *vps34::TRP1*) [49]. For *ARF1*: NYY0-2 (*MAT*α *ade2::ARF1::ADE2 arf1::HIS3 arf2::HIS3 ura3 lys2 trp1 his3 leu2)* and *arf1-11*^{ts}: NYY11-2 (NYY0-2 *ade2::arf1-11::ADE2*) [34].

Cell Imaging

GFP-PH domain fusion proteins were visualized in cells growing at log phase (OD_{600} nm = 1) at 30°C, unless otherwise stated. Cells were collected by brief centrifugation (2000 $\times$ *g*, 20 s), and 0.25 μl of resuspended cell pellet gently spread on a glass slide by application of a coverslip. Cells were examined immediately using a Radiance confocal microscope (Bio-Rad), and representative fields of cells were recorded. For immunofluorescent colocalization of GFP-chimeras, antibodies to TGN46 [50], Vti1a (Transduction Labs), or γ-adaptin (100/3, Sigma) in COS cells, or Tlg1p in yeast [51] were detected using appropriate Alex-568 secondary antisera (Molecular Probes).

Liposome Centrifugation Assays

Dioleoylphosphatidylcholine (DOPC, SIGMA) alone or mixed in a molar ratio of 39:1 with PtdIns(4)P or PtdIns(4,5)P_2 (Avanti Polar Lipids) were made up in 95% chloroform/5% methanol and dried under nitrogen. Lipids films were rehydrated in 250 mM raffinose, 25 mM HEPES, 1 mM DTT (pH 7.4), and extruded repeatedly through a polycarbonate membrane, pore size 0.2 μm (Whatman), to produce optically clear suspensions of small unilamellar liposomes. Liposomes were diluted with three volumes of Binding Buffer (BB) (125 mM KCl, 25 mM HEPES, 1 mM DTT, 1 mg/ml soybean trypsin inhibitor [pH 7.4]), pelleted by centrifugation at 50,000 rpm for 10 min (S120AT2, Sorvall), and gently resuspended in BB to yield a stock with final total lipid concentration of 25.6 mM. His_6-tagged GFP-PH domain chimeric proteins were purified to $\geq$80% homogeneity as described previously and diluted in BB to a final concentration of $\sim$50 μg/ml (1.1 μM). Reactions, consisting of 25 μl of liposomes diluted in BB and 25 μl protein solution, were incubated for 1 hr at 20°C and centrifuged at 50,000 rpm for 10 min (S100AT3, Sorvall). The GFP content remaining in the top 15 μl of the supernatant was measured in a LS50 spectrophotometer (excitation 485 nm, emission 515 nm, slit widths 5 nm). Phosphoinositides were assumed to be equally distributed between the two halves of the bilayer. Calculations of the Kd of PH binding to phosphoinositides were adjusted to take into account the finding that the asymptote of binding with increasing liposomes was reproducibly above zero on the *y* axis. Coomassie staining and Western blot analysis of proteins fractionated by SDS-PAGE revealed this to be due to cleavage of a proportion of each chimera (typically 20%) separating GFP and PH domain, with GFP-tagged and free PH domain being selectively recovered in liposome pellets and untagged GFP being found in the supernatants of binding reactions (data not shown).

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