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Svp1p defines a family of phosphatidylinositol 3,5-bisphosphate effectors

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Phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P_2), made by Fab1p, is essential for vesicle recycling from vacuole/lysosomal compartments and for protein sorting into multivesicular bodies. To isolate PtdIns(3,5)P_2 effectors, we identified *Saccharomyces cerevisiae* mutants that display *fab1*Δ-like vacuole enlargement, one of which lacked the *SVP1*/YFR021w/*ATG18* gene. Expressed Svp1p displays PtdIns(3,5)P_2 binding of exquisite specificity, GFP-Svp1p localises to the vacuole membrane in a Fab1p-dependent manner, and *svp1*Δ cells fail to recycle a marker protein from the vacuole to the Golgi. Cells lacking Svp1p accumulate abnormally large amounts of PtdIns(3,5)P_2. These observations identify Svp1p as a PtdIns(3,5)P_2 effector required for PtdIns(3,5)P_2-dependent membrane recycling from the vacuole. Other Svp1p-related proteins, including human and *Drosophila* homologues, bind PtdIns(3,5)P_2 similarly. Svp1p and related proteins almost certainly fold as β-propellers, and the PtdIns(3,5)P_2-binding site is on the β-propeller. It is likely that many of the Svp1p-related proteins that are ubiquitous throughout the eukaryotes are PtdIns(3,5)P_2 effectors. Svp1p is not involved in the contributions of *FAB1*/PtdIns(3,5)P_2 to MVB sorting or to vacuole acidification and so additional PtdIns(3,5)P_2 effectors must exist.

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Introduction

Phosphorylated derivatives of inositol and phosphatidylinositol fulfil a striking variety of specific functions in eukaryote cells, with their actions executed by effector proteins containing phosphoinositide-specific binding domains. Diverse protein modules can serve as phosphoinositide 'sensors'. These include many PH, FYVE and PX (≡PhoX) domains (Stenmark and Aasland, 1999; Lemmon and Ferguson, 2000; Gillooly *et al*, 2001; Ellson *et al*, 2002).

Phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P_2) is the most recently identified phosphatidylinositol bisphosphate isomer (Dove *et al*, 1997; Whiteford *et al*, 1997). All eukaryotes make PtdIns(3,5)P_2 using PtdIns3*P* 5-kinases related to *Saccharomyces cerevisiae* Fab1p (Cooke *et al*, 1998; Gary *et al*, 1998; Ikonomov *et al*, 2001). PtdIns(3,5)P_2 is essential for membrane recycling from the vacuole/lysosomes (Gary *et al*, 1998; Ikonomov *et al*, 2001; Dove *et al*, 2002), for ubiquitin-dependent packaging of proteins into multivesicular bodies (MVBs) (Odorizzi *et al*, 1998; Dove *et al*, 2002), for growth at high temperature (Yamamoto *et al*, 1995; Cooke *et al*, 1998; Gary *et al*, 1998) and for vacuole acidification (Bonangelino *et al*, 1997). Vac14p and Vac7p are Fab1p regulators (Gary *et al*, 1998; Bonangelino *et al*, 2002; Dove *et al*, 2002), and PtdIns(3,5)P_2 effector proteins that may facilitate MVB protein sorting have recently been identified (Friant *et al*, 2003; Whitley *et al*, 2003). Since deletion of these effectors does not lead to all the defects associated with loss of Fab1p, additional effectors remain to be identified. Other proteins that can bind PtdIns(3,5)P_2 (Xu *et al*, 2001; Cozier *et al*, 2002) seem unlikely to mediate any of the known effects of this lipid.

A single unlobed vacuole that largely fills the cell is a hallmark of *fab1*Δ yeast that cannot make PtdIns(3,5)P_2 (Yamamoto *et al*, 1995) so the loss of proteins that are Fab1p activators or are needed for the actions of PtdIns(3,5)P_2 should cause a similar phenotype. Using a microscopic screen starting from this vacuole phenotype, we sought genes whose disruption phenocopies the *fab1*Δ vacuole enlargement and identified the Fab1p regulator Vac14p/Svp2p (Dove *et al*, 2002).

Herein we show that Svp1p, another gene identified in this screen, is a specific PtdIns(3,5)P_2-binding protein that participates in the recycling of membrane proteins from the vacuole to the late endosome. Svp1p is the prototype member of a new family of phosphoinositide effectors.

Results

*svp1*Δ *cells have a fab1*Δ*-like vacuole defect*

Each Euroscarf strain lacks one non-essential gene. We screened these for enlarged vacuoles resembling those of *fab1*Δ cells, and provisionally termed the identified genes *SVP* (Swollen Vacuole Phenotype) (Dove *et al*, 2002).

The YFR021w open reading frame (ORF) encodes *SVP1*, which is now termed *ATG18* (Klionsky *et al*, 2003). Figure 1A shows DIC images of wild-type, *fab1*Δ and *svp1*Δ cells, and fluorescence images of the same cells with FM4-64-stained vacuoles. Most *fab1*Δ and *svp1*Δ cells are markedly enlarged, and both mutants usually have one large vacuole that fills much of the cell interior: normal vacuoles are smaller and multilobed. Remarkably, *SVP1* is physically separated from *FAB1* on chromosome VI by only one ORF (Figure 2A). *FAB1* overexpression does not correct the *svp1*Δ vacuole enlargement, so suppressed Fab1p expression cannot be its cause (not shown).

GFP-Svp1p rescues the vacuole defects of svp1Δ cells

GFP-Svp1p expression from the repressed MET25 promoter (with 5 mM methionine) corrected the *svp1*Δ vacuole enlargement. Rescue was variable and occurred at very low Svp1p expression (Figure 1B). *SVP1* disruption therefore causes the vacuole defects of *svp1*Δ cells. However, overexpressed GFP-Svp1p (methionine-free medium) disrupted vacuole function (Figure 1B). Both observations indicate that Svp1p has a role in regulating vacuole morphology.

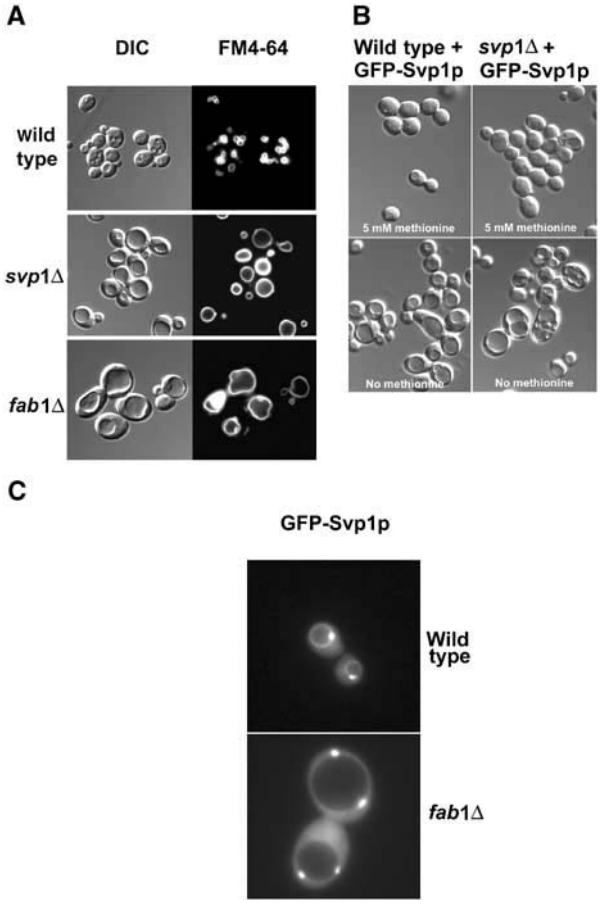


Figure 1 The *svp1*Δ phenotype involves vacuole enlargement, and GFP-Svp1p localisation at the vacuole membrane is *FAB1*-dependent. (**A**) Differential interference contrast images and FM4-64 staining of wild-type, *svp1*Δ and *fab1*Δ cells, demonstrating the greatly enlarged vacuoles of *svp1*Δ cells. (**B**) Svp1p expression corrects the *svp1*Δ vacuolar defect (upper images, taken during methionine-repressed low-level Svp1p expression), but Svp1p overexpression induces cell vacuolation (lower images, during derepressed expression following methionine removal). (**C**) When GFP-Svp1p was expressed in *svp1*Δ cells, it associated mainly with the vacuole membrane and large punctate structures. Little or none of the GFP-Svp1p was associated with the vacuole membrane in *fab1*Δ cells.

GFP-Svp1p localises to the vacuole membrane in a FAB1-dependent manner

Phosphoinositide-binding proteins frequently show inositol lipid-dependent changes in intracellular localisation. When GFP-Svp1p was expressed at low levels in wild-type cells, it localised to the vacuole membrane and to a punctate compartment (Figures 1C and 4B). In contrast, most *fab1*Δ cells lacked vacuole-associated GFP-Svp1p (Figure 1C), but the GFP-Svp1p on the punctate compartment remained (Figure 1C). Localisation of GFP-Svp1p to the vacuole membrane, but not to the punctate compartment, is therefore Fab1p-dependent, as would be expected for a PtdIns(3,5)P_2 effector.

Previous work on the SVP1 gene

SVP1 has previously been named *ATG18*, *AUT10* and *CVT18*, reflecting its involvement in AUTophagy, and in Cytoplasm-to-Vacuole protein Targeting (Barth *et al*, 2001; Guan *et al*, 2001).

None of these studies mentioned vacuole enlargement, possibly because they examined *svp1*Δ cells under starvation conditions that tend to provoke vacuole enlargement even in wild-type cells.

Svp1p-like proteins are present in all eukaryotes

The *S. cerevisiae* genome encodes two other *SVP1*-like proteins, YPL100w/*MAI1* and YGR223c (Georgakopoulos *et al*, 2001; Barth *et al*, 2002), which we term *HSV1* and *HSV2* (Homologous with *SVP1*), respectively. Their disruption did not cause vacuole enlargement (not shown). GFP-Hsv1p/Mai1p and GFP-Hsv2p localised to a non-vacuolar punctate compartment and this localisation did not require *FAB1* (not shown).

Svp1p-like proteins are widespread in all eukaryotes (Figure 2B; Barth *et al*, 2001). For example, the human and *Arabidopsis* genomes both encode at least three, and *Caenorhabditis elegans* and *Drosophila melanogaster* have two or more (Barth *et al*, 2001). Gene DKFZp434J154 encodes the most Svp1-like human homologue (labelled hSvp1a in Figures 2B and C).

Svp1p and its homologues are multi-WD40 proteins that fold as β-propellers

SVP1 encodes a serine-rich 500-amino-acid protein with two previously recognised WD-40 motifs near the centre of the sequence (residues 234–274 and 277–318, within blades 5 and 6 in Figure 2C) (Barth *et al*, 2001; Guan *et al*, 2001). The 3D-PSSM fold recognition server (Kelley *et al*, 2000) predicts that Svp1p, hSvp1a and Hsv2p fold as seven-bladed β-propellers. They show high similarity scores when compared with known seven-bladed β-propellers—the transducin β-subunit and the C-terminal domain of Tup1p (Figure 2C). Trypsin cleaves Svp1p at Arg377 (Figures 2B and D, black arrow), yielding Svp1p$^{1-377}$ as a stable fragment (MC King and MA Lemmon, unpublished). Combining the results from the threading analysis and trypsin cleavage, we suggest that the Svp1p β-propeller ends around R377, and that the

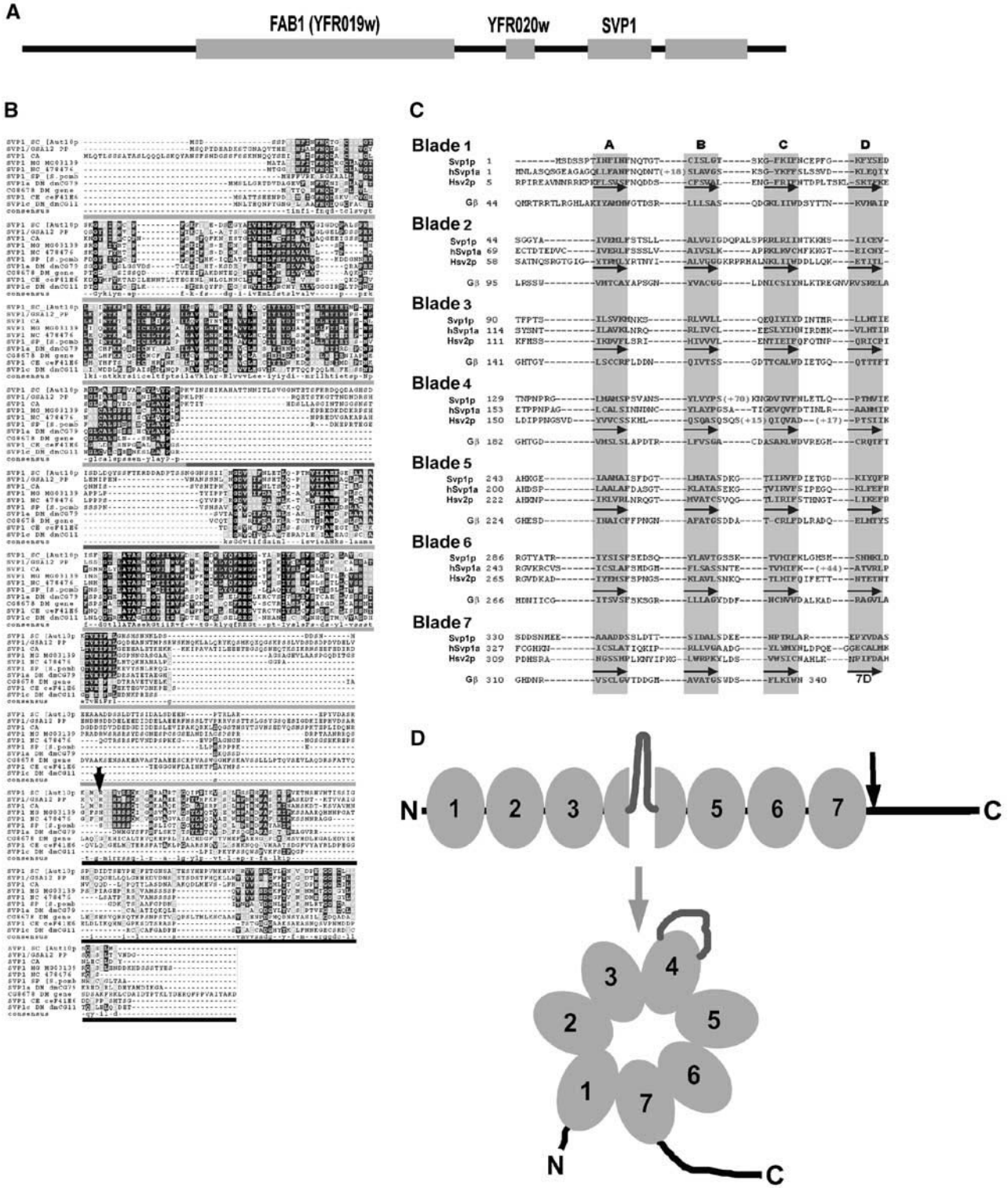


Figure 2 Sequence analysis and structural modelling of Svp1p and Svp1p-like proteins. (**A**) YFR021w/*SVP1* is on the right arm of chromosome VI, adjacent to *FAB1*. (**B**) ClustalW alignment of some eukaryotic Svp1-like proteins. Light-grey bars identify sequence within the putative β-propeller. The dark-grey bar denotes the B/C insert in blade 4. The black arrow shows the deduced site of trypsin cleavage in Svp1p. The black bar indicates the C-terminal domain outside the β-propeller. (**C**) Threaded alignment of Svp1p, hSvp1a and Hsv2p with the β-propeller of transducin-β (Sondek *et al*, 1996). Assignment of blades and β-strands is based on transducin-β. Each sequence was submitted to the 3D-PSSM server (Kelley *et al*, 2000), and gave a significant (>80% certainty) score for alignment with transducin-β. Alignments were slightly adjusted in blades 3, 4 and 7, to maintain consistency within the Svp1p family ClustalW alignments. (**D**) A linear depiction of the Svp1p domain structure and a cartoon of its probable folded structure. Light ovals represent WD-40 blades, and the black arrow the point of trypsin attack.

C-terminal 123 residues form an independent domain (see Figure 2D).

Sequence alignment (Figure 2C) shows that the predicted β-strands of the propeller blades are quite well conserved between Svp1p and its homologues, but the intervening loops are highly divergent. For instance, most homologues lack the large B/C loop in blade 4 of Svp1p (Figures 2B–D; Barth *et al*, 2001). The C-terminal domains are also highly variable.

Svp1p, Hsv1p, Hsv2p and hSvp1a bind PtdIns(3,5)P_2 with high affinity and specificity

GFP-Svp1p localises to the vacuole in a *FAB1*-dependent manner, so we determined whether Svp1p might bind PtdIns(3,5)P_2, particularly under ionic conditions like those in the cytosol (inc. millimolar Mg^{2+}). We first used phosphoinositide 'dot blots' (Kavran *et al*, 1998) and saw binding of ^{32}P-labelled GST-Svp1p (Figure 3A) and GST-Hsv2p (not shown) only to PtdIns(3,5)P_2 and PtdIns3*P*.

Since dot-blot assays do not always report a protein's native lipid selectivity reliably, we used two more quantitative approaches to analyse phosphoinositide binding by GST-Svp1p. First, we found that GST-Svp1p bound strongly to Affigel beads bearing covalently attached PtdIns(3,5)P_2 (Figure 3B, 'total binding'). Washing with buffer containing PtdIns(3,5)P_2 almost completely displaced the bound GST-Svp1p, but other PtdIns*P* and PtdInsP_2 isomers had no effect (Figure 3B). Gro*P*Ins(3,5)P_2, the hydrophilic backbone of PtdIns(3,5)P_2, did not displace Svp1p (not shown).

Specific binding of PtdIns(3,5)P_2 to Svp1p therefore involves at least two interactions: (a) between the anionic head group and a basic amino-acid cluster; and (b) between hydrophobic parts of the lipid and a nearby hydrophobic patch on Svp1p.

Surface plasmon resonance (SPR) analysis was undertaken to obtain a quantitative measure of Svp1p binding to mixed lipid vesicles 'doped' with a phosphoinositide (3%, mol/mol) and immobilised on BIACore L1 chips (Yu and Lemmon, 2001). GST-Svp1p bound strongly to PtdIns(3,5)P_2-containing membranes in the presence of 0.5–2 mM $MgCl_2$ (apparent $K_D \sim 180$ nM; Figure 3G). Since GST-induced dimerisation will potentiate Svp1p binding to phosphoinositides, we also examined native Svp1p that was monomeric by gel filtration. This bound PtdIns(3,5)P_2 selectively and with high affinity (half-maximal at ~500 nM Svp1p) (Figures 3C and D). Svp1p bound weakly to PtdIns3*P*, and not to PtdIns(4,5)P_2, PtdIns(3,4)P_2 or PtdIns4*P*.

We compared PtdIns(3,5)P_2 binding by Svp1p with the interactions between other highly selective phosphoinositide sensor domains and their phosphoinositide ligands (Figure 3D). The affinity of the Svp1p/PtdIns(3,5)P_2 interaction was at least 10-fold greater than between the Hrs1 FYVE domain and PtdIns3*P* (Sankaran *et al*, 2001), higher than for the phospholipase C-δ_1 PH domain/PtdIns(4,5)P_2 interaction (Rebecchi and Pentyala, 2000), and weaker than for PtdIns(3,4)P_2 binding to the DAPP1 PH domain (Ferguson *et al*, 2000) (Figure 3D).

The PtdIns(3,5)P_2 selectivity of Svp1p was reduced when the buffer lacked Mg^{2+}. Although the PtdIns(3,5)P_2 affinity was enhanced ($K_D \sim 18$ nM), Svp1p also bound to PtdIns(3,4)P_2 ($K_D \sim 190$ nM) and PtdIns(4,5)P_2 ($K_D \sim 200$ nM) (Figures 3E and F), although less avidly and with one-third as much bound at saturation, possibly indicating an altered stoichometry.

GST-Hsv2p and GST-hSvp1a bound to PtdIns(3,5)P_2 with high affinity (Figure 3G; 0.5 mM $MgCl_2$ present) and selectivity (not shown): the affinity of GST-Hsv2p was similar to that of GST-Svp1p. GST-Hsv1p/Mai1p also bound specifically to PtdIns(3,5)P_2 ($K_D \sim 500$ nM), as did a GST-Tagg-340-residue *Drosophila* Svp1p homologue with a very short C-terminal tail (CG11975 in Figure 2B; K_D 200–500 nM) (not shown).

It therefore appears that specific PtdIns(3,5)P_2 binding is a conserved and functionally important feature of many Svp1p-like proteins.

PtdIns(3,5)P_2 binds to the β-propeller

The PtdIns(3,5)P_2-binding site of Svp1p does not require the C-terminal sequence that lies outside the β-propeller: its removal did not change PtdIns(3,5)P_2 binding. However, Svp1p$^{1-170}$ and Svp1p$^{171-500}$, each of which includes about half of the proposed β-propeller, did not bind PtdIns(3,5)P_2. Some Svp1p homologues have quite large insertions within the β-propeller, for example, between strands 4B and 4C (Figures 2B–D). An Svp1p construct lacking this loop (Svp1p$^{\Delta 4B/C}$) also bound PtdIns(3,5)P_2 with wild-type specificity and affinity (Figure 4A).

It seems that PtdIns(3,5)P_2 binds to the β-propeller. A multiple alignment highlighted two clusters of basic residues that might be involved. There is a widely conserved basic sequence at the junction between blades 5 and 6 (QFRRG in Svp1p) that includes an invariant RRG, and the basic character of a sequence at the start of strand 2C (SPRRLR in Svp1p) is also widely conserved (Figure 2C).

Mutating $_{284}$F**RR**G$_{287}$ to $_{284}$F**TT**G$_{287}$ (Svp1p^{FTTG}) decreased the PtdIns(3,5)P_2 affinity more than 40-fold (Figure 4A). By contrast, changing $_{71}$SP**RR**L**R**$_{76}$ to $_{71}$SP**SS**L**S**$_{76}$ (Svp1p^{SPSSLS}) only slightly reduced PtdIns(3,5)P_2 binding (Figure 4A).

Svp1p constructs only correct the svp1Δ vacuole enlargement if they bind PtdIns(3,5)P_2

GFP-Svp1p^{FTTG} expressed in yeast was present in the cytosol and nucleus—none was vacuole-associated (Figure 4B). In contrast, GFP-Svp1p^{SPSSLS} localised to the vacuole normally (Figure 4B).

Expression of Svp1p^{FTTG} under control of its own promoter did not correct the vacuole enlargement in *svp1*Δ cells (not shown), suggesting that Svp1p must bind PtdIns(3,5)P_2 if it is to function normally in vacuole membrane trafficking. Unexpectedly, though, overexpressing GFP-Svp1p^{FTTG} in wild-type yeast provoked a vacuole enlargement like that caused by wild-type Svp1p (Figure 4C). This suggests that GFP-Svp1p^{FTTG} remains capable of sequestering something, probably a soluble protein, that is needed for retrograde membrane trafficking, but cannot deliver it to the PtdIns(3,5)P_2-rich vacuole.

Membrane recycling from the vacuole fails in svp1Δ cells

Vacuole enlargement in *fab1*Δ, *vac7*Δ and *vac14/svp2*Δ cells is caused, at least partially, by a failure of membrane recycling to the late endosome (R Piper, unpublished) (Bryant *et al*, 1998). As explained in Figure 5A, this process can be detected *in vivo* by appearance in the Golgi of proteolytically

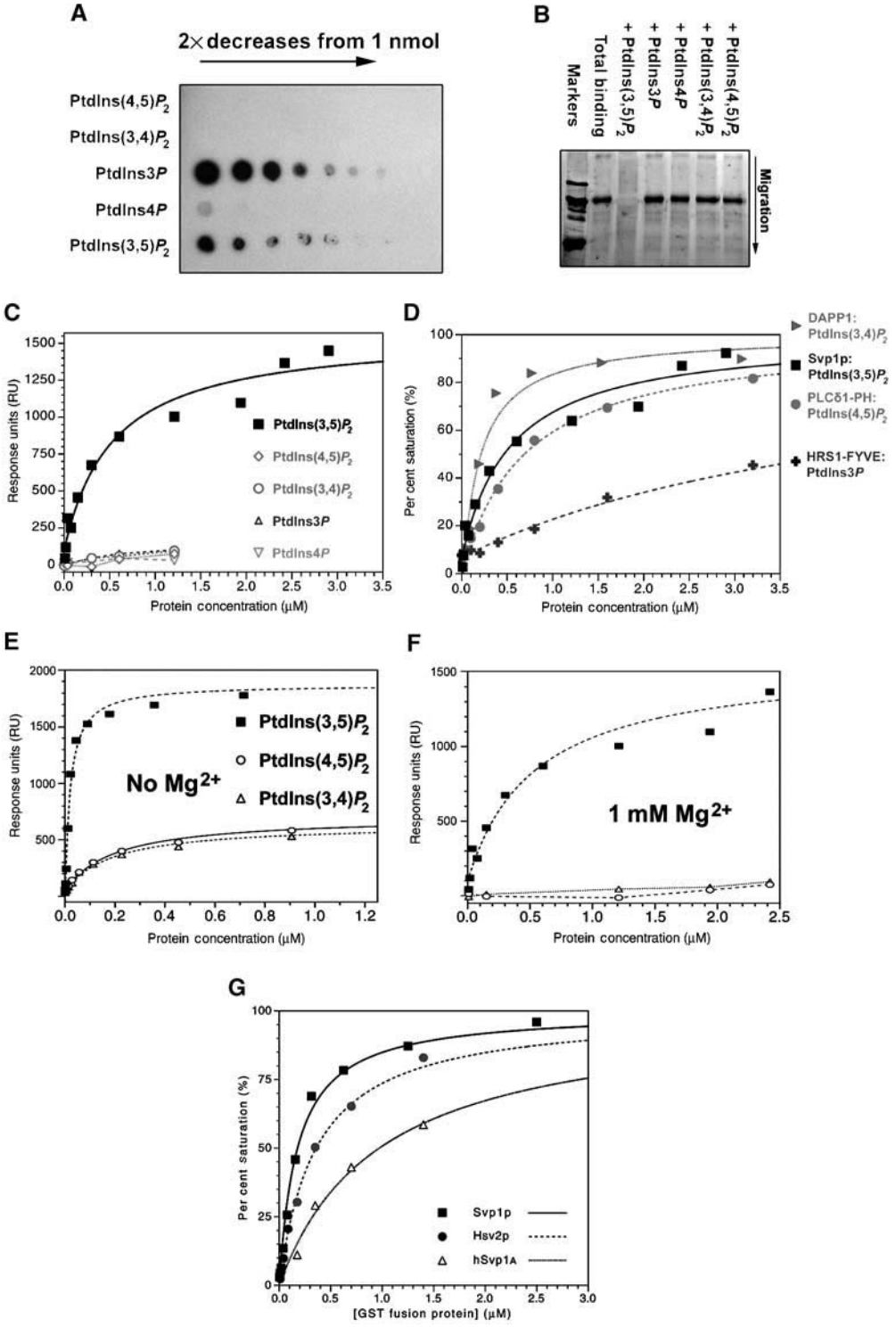


Figure 3 Svp1p and related proteins bind PtdIns(3,5)P_2 with high affinity and selectivity. (**A**) A dot-blot assay indicates that GST-Svp1p binds both PtdIns3*P* and PtdIns(3,5)P_2. (**B**) GST-Svp1p bound to PtdIns(3,5)P_2-derivatised Affigel beads is selectively displaced by exogenous PtdIns(3,5)P_2 and not by other PtdinsP_2 isomers or by PtdIns*P*s. (**C**) Monomeric Svp1p binds to a DOPC layer 'doped' with 3 mol% PtdIns(3,5)P_2 but not with other phosphoinositides, as detected by Biacore analysis. (**D**) The affinity of monomeric Svp1p binding to PtdIns(3,5)P_2-'doped' lipids is similar to phosphoinositide affinities in other protein/phosphoinositide combinations. (**E, F**) Mg^{2+} at a 'physiological' (0.5 mM) concentration is needed for Svp1p to show its full PtdIns(3,5)P_2 selectivity. (**G**) Comparison of PtdIns(3,5)P_2 binding by GST-Svp1p, GST-Hsv2p (*S. cerevisiae*) and GST-hSvp1a (human). All data are representative of at least three independent experiments.

matured RS-ALP (mRS-ALP), a variant of the Pho8p alkaline phosphatase that has an FXFXD motif incorporated into its N-terminal region. In stage 1, recently synthesised pro-RS-ALP traffics from the Golgi directly to the vacuole, via an AP-3-dependent route. It is processed to mRS-ALP by vacuolar Pep4p (stage 2), and the FXFXD motif then directs mRS-ALP into the *FAB1*/PtdIns(3,5)P_2-dependent retrograde pathway from the vacuole to late endosomes (stage 3), from whence it

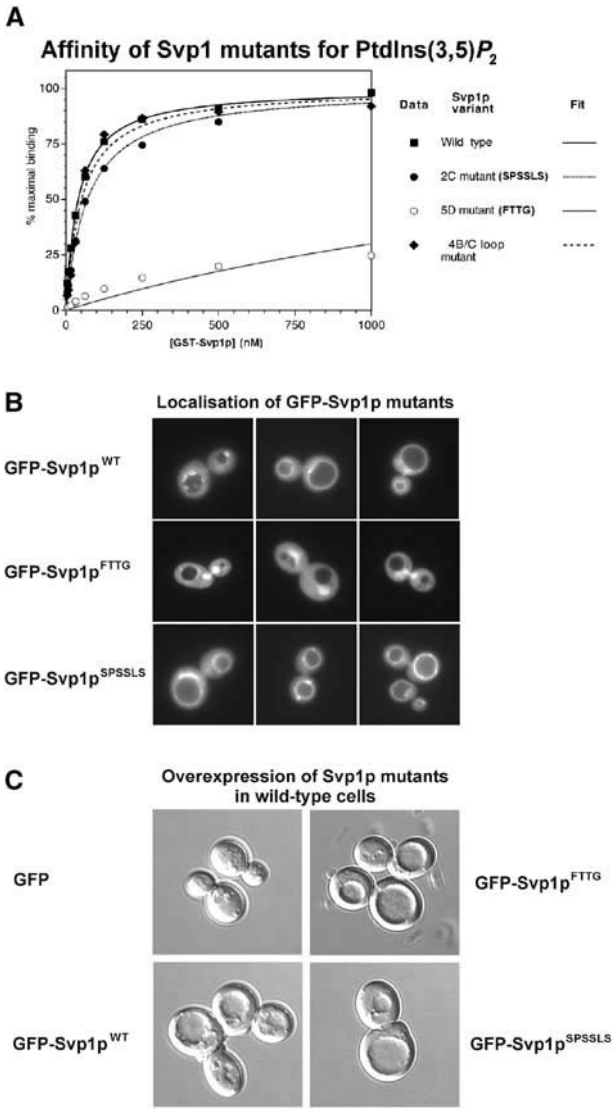


Figure 4 The β-propeller of Svp1p binds PtdIns(3,5)P_2. (**A**) PtdIns(3,5)P_2 binding to GST-Svp1p mutants lacking the blade 4 B/C loop or with a mutated basic patch in β-sheet 2C (SPRRLR to SPSSLS) or β-sheet 5D (FRRG to FTTG). Only the conversion of Arg residues to Thr in the blade 5 basic patch substantially curtailed PtdIns(3,5)P_2 binding. (**B**) Localisation of the Svp1p mutants in *svp1*Δ yeast. Wild type and Svp1p^{SPSSLS} localised similarly, but Svp1p^{FTTG} is no longer on vacuole membranes. The constructs were expressed as N-terminal GFP fusions from a single-copy pUG36 plasmid under control of the MET25 promoter, with 0.3 mM methionine. (**C**) Despite not associating with the vacuole membrane, overexpressed GFP-Svp1p^{FTTG} causes vacuolation of 60–70% of wild-type cells (compared with 60–70% of cells when GFP-Svp1p^{WT} is overexpressed). The constructs are N-terminal GFP fusions in pUG36, and were grown without methionine for maximal expression.

goes to the Golgi via the retromer pathway (stage 4). As a result, the wild-type Golgi contains both pro-RS-ALP and mRS-ALP, but no mRS-ALP gets to the Golgi of cells defective in step 3 (e.g. *fab1*, *vac7*, *vac14* and *pep12* mutants).

We detected both pro-RS-ALP and mRS-ALP in Golgi membranes from wild-type cells, but the *svp1*Δ Golgi contained no mRS-ALP (Figure 5B). This demonstration of a failure of mRS-ALP recycling, together with the vacuole enlargement in *svp1*Δ cells, suggests that the initial step

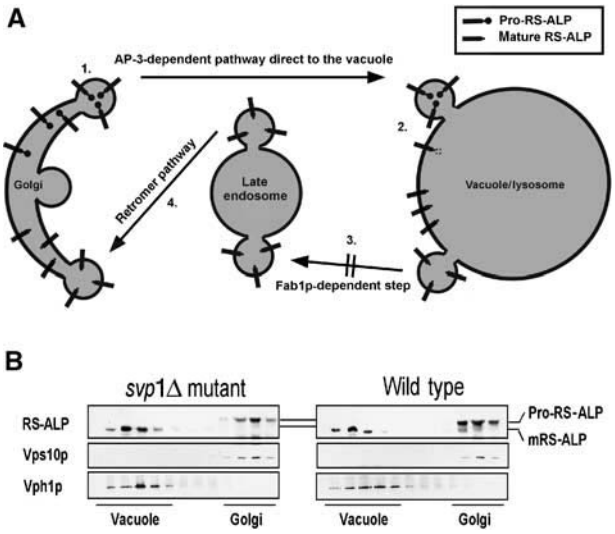


Figure 5 Svp1p is needed for the recycling of vacuole membrane proteins. (**A**) Scheme depicting routes of trafficking of RS-ALP trafficking (for explanation, see the text). (**B**) In *svp1*Δ cells, the Golgi contains only pro-RS-ALP. Vph1p serves as a marker for the vacuole membrane, and Vps10p for Golgi membrane (for details, see Materials and methods).

that launches membrane into the vacuole-to-late-endosome retrograd trafficking pathway requires the Svp1p/PtdIns(3,5)P_2 complex.

*svp1*Δ *cells make abnormally large amounts of PtdIns(3,5)*P_2

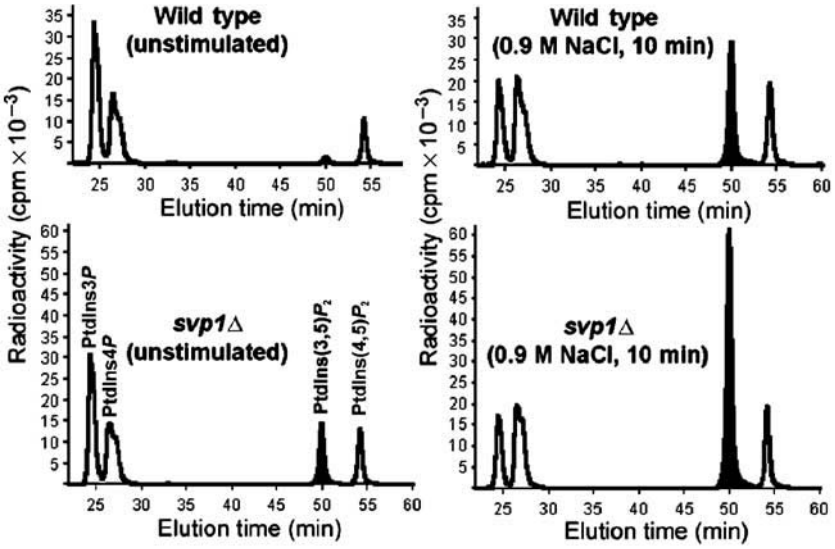
The above observations indicate that some Svp1p functions rely on Fab1p-catalysed PtdIns(3,5)P_2 production, so we checked that *svp1*Δ cells can make PtdIns(3,5)P_2. Lipids were extracted from unstressed ^{3}H-inositol-labelled cells and from cells that were salt-stressed to provoke rapid PtdIns(3,5)P_2 synthesis (Dove *et al*, 1997).

Remarkably, unstressed *svp1*Δ cells contained 5–10 times more PtdIns(3,5)P_2 than wild type: PtdIns4*P*, PtdIns3*P* and PtdIns(4,5)P_2 levels were normal (Figures 6A and B). There was four times more PtdIns(3,5)P_2 than PtdIns(4,5)P_2 in salt-challenged *svp1*Δ cells—making PtdIns(3,5)P_2 comprise 6% of the phosphoinositides, more than has been seen in any other cell. The fact that vacuole defects persist in *svp1*Δ cells that contain exorbitant amounts of PtdIns(3,5)P_2 underlines the fact that *svp1*Δ cells do not respond appropriately to PtdIns(3,5)P_2.

Deletion of *HSV1* or *HSV2*, or both, did not change the cellular PtdIns(3,5)P_2 complement (not shown).

Svp1p is not a PtdIns(3,5)P_2 *phosphatase*

Might PtdIns(3,5)P_2 accumulate in *svp1*Δ cells because the missing Svp1p is a PtdIns(3,5)P_2 phosphatase? Although the Svp1p sequence includes no recognisable phosphatase-like motifs, we compared the ability of biologically active GST-Svp1p to hydrolyse PtdIns(3,5)P_2 with that of the PtdIns(3,5)P_2 3-phosphatase MTMR3 (Walker *et al*, 2001). Under conditions in which GST-MTMR3 rapidly dephosphorylated PtdIns(3,5)P_2, GST-Svp1p showed no activity (not shown). Moreover, wild-type cells that overexpressed GFP-Svp1p retained a normal PtdIns(3,5)P_2 complement and



	^{3}H (dpm×10^{-3})			
	Wild type		Δ*svp1*	
	Basal	High salt	Basal	High salt
PtdIns	7050	6960	6890	6640
PtdIns3*P*	250	120	270	130
PtdIns4*P*	170	195	190	230
PtdIns(3,5)P_2	**11**	**175**	**115**	**435**
PtdIns(4,5)P_2	70	110	97	120

Figure 6 *svp1*Δ cells accumulate abnormally large amounts of PtdIns(3,5)P_2. Anion-exchange HPLC chromatograms of the PtdIns*P* and PtdInsP_2 complements of wild-type and *svp1*Δ cells, and their responses to hyperosmotic stress. The deacylated phosphoinositides eluted in the order: PtdIns3*P*, PtdIns4*P*, PtdIns(3,5)P_2 (filled peak) and PtdIns(4,5)P_2 (see lower left panel). The relative amounts of phosphoinositides in *svp1*Δ cells are also shown in the table. The total phosphoinositide complement is unchanged, but in *svp1*Δ cells an abnormally large proportion of this is PtdIns(3,5)P_2. Cells were labelled to isotopic equilibrium, so relative phosphoinositide concentrations match the relative levels of labelling. The data are representative of at least four experiments.

showed a normal increase in PtdIns(3,5)P_2 content following hyperosmotic stress (not shown).

The autophagic role of Svp1p does not need FAB1 or PtdIns(3,5)P_2

The observation that *svp1*Δ cells fail to initiate autophagy correctly (Barth *et al*, 2001) led to its previous designation as *AUT10*. To determine whether Svp1p must bind PtdIns(3,5)P_2 to play its part in autophagy, we investigated this process in *fab1*Δ cells, which express Svp1p but contain no PtdIns(3,5)P_2.

We assessed autophagy by following the maturation of a truncated Pho8p pro-enzyme (Pho8Δ60p) to active alkaline phosphatase. Pho8Δ60p lacks a transmembrane domain needed for trafficking to the vacuole and is made as an inactive cytosolic protein. Its processing needs intravacuolar Pep4p, so it only becomes activated when bulk-sequestered cytosol is transferred into the vacuole during starvation-induced autophagy (Noda *et al*, 1995; Huang and Klionsky, 2002; Noda *et al*, 2002).

Pho8Δ60p trafficking was suppressed in *svp1*Δ cells (Figure 7A) to the same degree as in cells lacking Apg1p, another essential protein (Harding *et al*, 1996; Matsuura *et al*, 1997; Abeliovich *et al*, 2003). However, Pho8Δ60p matured normally in *fab1*Δcells, so Svp1p does not need to interact with Fab1p and/or PtdIns(3,5)P_2 to fulfil its role in autophagy.

Some FAB1/PtdIns(3,5)P_2-dependent functions do not require Svp1p, Hsv1p or Hsv2p

Heat tolerance. *fab1*Δ yeast grow poorly and lyse at elevated temperatures (Yamamoto *et al*, 1995) (Figure 7B). However, *svp1*Δ cells were almost as heat-tolerant as wild-type cells, and their slight sensitivity at 44°C was ameliorated by simultaneous deletion of *HSV1* and *HSV2* (data not shown). None of the Svp1p-like proteins is therefore needed for PtdIns(3,5)P_2 to confer heat tolerance to cells.

Vacuole acidification. Inactivation of *VAC14/SVP2, VAC7* or *FAB1* causes a failure of vacuole acidification, even though the vacuolar H^+-ATPase (V-ATPase) localises correctly. It has been suggested that PtdIns(3,5)P_2 might positively regulate V-ATPase or somehow stabilise the proton gradient (Bonangelino *et al*, 1997; Gary *et al*, 2002).

Quinacrine, which is fluorescent and accumulates in acidified intracellular compartments, was used to show the severe

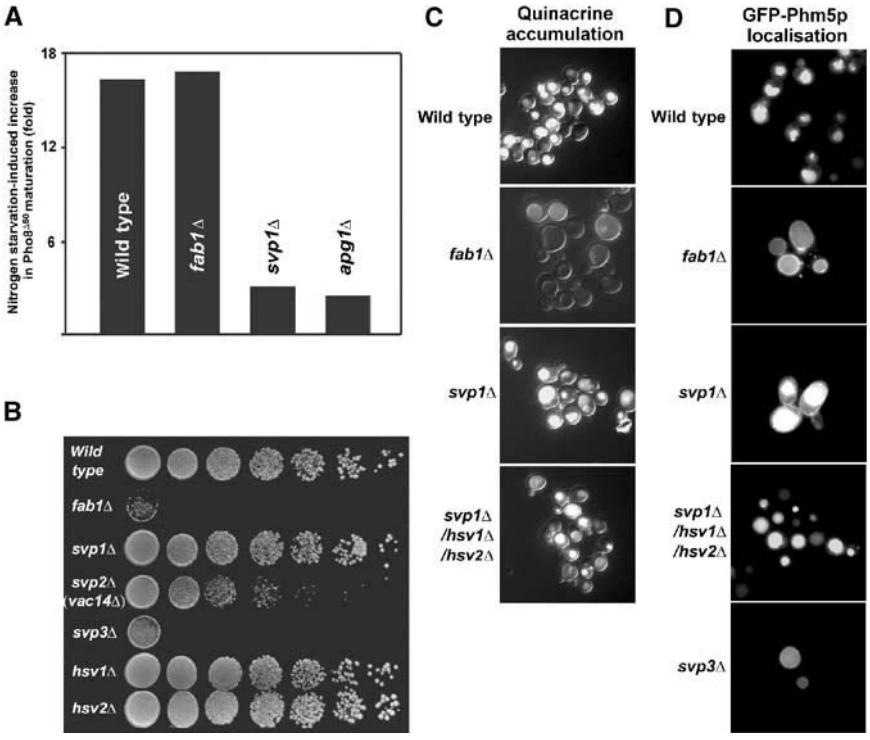


Figure 7 The role of Svp1p in autophagy is PtdIns(3,5)P_2-independent, and *S. cerevisiae* Svp1p-like proteins are not needed for several *FAB1*/PtdIns(3,5)P_2-dependent processes. (**A**) Autophagy processed Pho8Δ60p normally in *fab1*Δ cells, but not in *svp1*Δ cells or in the autophagy mutant *apg1*Δ (for details, see Materials and methods). (**B**) Svp1p-related proteins are not needed to maintain growth at elevated temperatures (for details, see Materials and methods). All strains grew at 23°C, so only the 42°C plate is shown. Data are representative of those from three or four experiments that gave similar results. (**C**) Svp1p-related proteins are not needed for vacuole acidification, as assessed by accumulation of the fluorescent weak base quinacrine. (**D**) Svp1p-related proteins are not needed for the sorting of proteins into MVB, as assessed by the trafficking of GFP-Phm5p.

acidification defect of *fab1*Δ cells (Gary *et al*, 1998). When we stained wild type, *fab1*Δ, *svp1*Δ and *svp1*Δ/*hsv1*Δ/*hsv2*Δ triple deletion mutants similarly, vacuole acidification was only compromised in the *fab1*Δ cells (Figure 7C). Control of acidification must therefore employ a PtdIns(3,5)P_2-dependent pathway that needs none of the known Svp1p-related proteins.

Protein sorting to the MVB. Cells that lack Fab1p or its activator Vac14p do not correctly sort certain proteins into MVBs, and irreversible ubiquitination of cargo proteins corrects this defect (Odorizzi *et al*, 1998; Dove *et al*, 2002).

This process can be monitored by assessing traffic to the vacuole of GFP-Phm5 (Dove *et al*, 2002). This normally goes into the lumen, but defective sorting mislocates it at the membrane. GFP-Phm5p sorting was normal in *svp1*Δ and triple-deletion *svp1*Δ*//hsv1*Δ/*hsv2*Δ cells, but defective in *fab1*Δcells (Figure 7D)—so none of the Svp1p-like proteins contribute to this PtdIns(3,5)P_2-dependent process.

Discussion

The first insight into the biology of PtdIns(3,5)P_2 came with the discovery that Fab1p is the PtdIns3*P* 5-kinase that makes this lipid (Cooke *et al*, 1998; Gary *et al*, 1998). Cells lacking *FAB1*, or expressing a kinase-inactive version of Fab1p in a *fab1*Δ background (F Cooke, unpublished), display a complex spectrum of dysfunctions, including vacuole enlargement, mis-sorting of proteins into MVBs, heat-sensitive cell lysis, faulty vacuole acidification and problems with vacuole inheritance (Yamamoto *et al*, 1995; Odorizzi *et al*, 1998; Dove *et al*, 2002). Why do *fab1*Δ cells display such a disparate set of dysfunctions, most or all of which are caused by a lack of PtdIns(3,5)P_2? Do cells have one PtdIns(3,5)P_2 effector protein that contributes to multiple cell functions, or does PtdIns(3,5)P_2 interact with several effectors, with each fulfilling a different function(s)?

With the aim of identifying PtdIns(3,5)P_2 targets, we screened the EUROFAN deletion mutant collection for gene deletions that cause *fab1*Δ-like vacuole enlargement (Dove *et al*, 2002). From this screen, YFR021w/*SVP1* was particularly intriguing—it is very near YFR019w/*FAB1* on chromosome VI and all eukaryotes have homologues.

PtdIns(3,5)P_2 specificity of Svp1p and related proteins

That GFP-Svp1p localises to the vacuole in a *FAB1*-dependent manner suggested that Svp1p might be a PtdIns(3,5)P_2 effector. However, Svp1p and Fab1p also seem to interact directly (Georgakopoulos *et al*, 2001), so that might also contribute to the vacuole localisation. Others have also concluded that some Svp1p is localised to a punctate compartment near the vacuole (Barth *et al*, 2001). Very gentle lysis disrupted the vacuole localisation of GFP-Svp1p but not its punctuate localisation (Guan *et al*, 2001), and our evidence also indicates that different mechanisms regulate Svp1p targeting to each compartment.

Under approximately 'physiological' conditions of ionic strength and Mg^{2+} concentration, monomeric Svp1p is a

very selective and high-affinity PtdIns(3,5)P_2-binder, as are two related yeast proteins (Hsv1p and Hsv2p), the *Drosophila* homologue CG11975 and the human homologue hSvp1a. This suggests that Svp1p and its homologues constitute a substantial family of PtdIns(3,5)P_2 specific effector proteins. These are the first proteins known to bind phosphoinositides through small basic amino-acid patches on a β-propeller structure, and so it is possible that a subset of the many proteins encoding β-propeller/WD-40 motifs may also bind to phosphoinositides. This localisation of the Svp1p PtdIns(3,5)P_2 binding on its β-propeller also suggests a possible structural analogy with the recently discovered binding of a phosphothreonine-containing peptide to the eight-bladed β-propeller of Cdc4p (Orlicky *et al*, 2003).

Cellular roles of Svp1p

The normal yeast vacuole comprises an array of large organelles that undergo homotypic fusion with other vacuole elements (Wickner, 2002) and heterotypic fusion with smaller organelles, including MVBs (Odorizzi *et al*, 1998). Following the recruitment of membrane by fusion, the vacuole and MVB elements may normally re-segregate in a manner similar to that seen in cell-free studies with mammalian lysosome/late endosome hybrid organelles (Luzio *et al*, 2000) or a vesicular intermediate may be involved in vacuole to late endosome trafficking. It is likely that Svp1p is involved in a PtdIns(3,5)P_2-dependent prebudding step in this vacuole membrane segregation process, as suggested in the model in Figure 8A. When this retrograde trafficking step is blocked, as in *fab1*Δ and *svp1*Δ cells, far more vacuole membrane accumulates than in normal cells and the multiple vacuole elements fuse (Yamamoto *et al*, 1995).

Studies of trafficking of the v-SNARE, Vti1p, have confirmed a defect in this segregation/reformation process when PtdIns(3,5)P_2 production is impaired. Vti1p is involved in the fusion of late endosomes/MVBs and the vacuole, and a retrograde pathway normally recycles Vti1p to late endosomes. This is blocked in *vac7*Δ cells (Bryant *et al*, 1998), which lack the Fab1p activator Vac7p and make little PtdIns(3,5)P_2 (Gary *et al*, 2002). Second mutations that restore PtdIns(3,5)P_2 to normal levels rescue the *vac7*Δ defects: for example, a suppressive mutation of *FIG4*, which encodes a phosphoinositide phosphatase (Gary *et al*, 2002). Our RS-ALP trafficking results show that *svp1*Δ cells phenocopy this *vac7*Δ defect, almost certainly because Svp1p is the PtdIns(3,5)P_2 effector required for this membrane recycling.

Our working hypothesis is that the Svp1p/PtdIns(3,5)P_2 complex participates in a specific interaction with some other protein (or complex), and so directs budding from the vacuole surface. Folded as a β-propeller, Svp1p is well suited to form a platform for protein–protein interactions, probably of the peptide-in-groove type (ter Haar *et al*, 2000). Our autophagy studies make it clear that Svp1p also performs PtdIns(3,5)P_2-independent vacuole-related functions, and involvement of the same binding partners in these functions might constitute a mechanistic link between the PtdIns(3,5)P_2-dependent and -independent functions of Svp1p (Barth *et al*, 2001; Guan *et al*, 2001).

Since *svp1*Δ cells accumulate abnormally large amounts of PtdIns(3,5)P_2, it also seems likely that the Svp1p/PtdIns(3,5)P_2 complex either restrains PtdIns(3,5)P_2 synthesis or activates PtdIns(3,5)P_2 degradation. The known interaction between Fab1p and Svp1p (Georgakopoulos *et al*, 2001) suggests a possible mechanism for feedback regulation of PtdIns(3,5)P_2 synthesis.

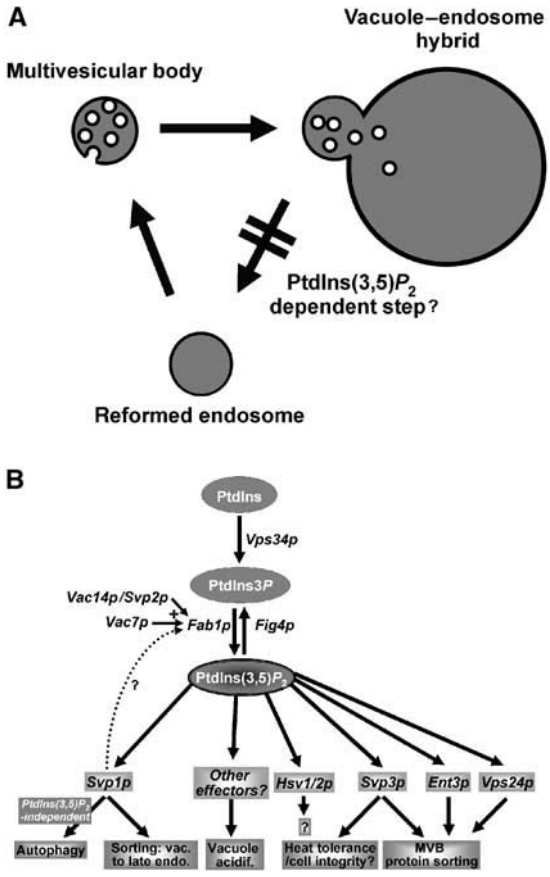


Figure 8 The involvement of Fab1p, PtdIns(3,5)P_2 and downstream effector proteins in yeast cell functions. (**A**) Outline of the cycle of vacuole membrane addition and retrieval for which PtdIns(3,5)P_2 appears to be essential. It is not clear whether retrograde vacuole-to-late-endosome trafficking occurs by re-segregation of the vacuole and late endosome or by the traffic of a vesicular intermediate between these structures. (**B**) A tentative synthesis of how the actions of multiple PtdIns(3,5)P_2 effector proteins may contribute to various cell functions (see Discussion). All of these, except the involvement of Svp1p in autophagy, require the presence in cells of *FAB1* and/or PtdIns(3,5)P_2.

Svp1p as one of several PtdIns(3,5)P_2 effectors?

Taken with recent reports that Ent3p, Ent5p and Vps24p are effectors that contribute to the MVB trafficking functions of PtdIns(3,5)P_2 (Friant *et al*, 2003; Whitley *et al*, 2003), our results support the idea that the complex *fab1*Δ phenotype is caused by malfunctions in several, probably independent, PtdIns(3,5)P_2 effector pathways. It is clear that neither Ent3p, Ent5p nor Vps24p (Friant *et al*, 2003; Whitley *et al*, 2003), nor any of the Svp1p-related proteins, participates in the effects of PtdIns(3,5)P_2 on vacuole acidification or heat tolerance. Elsewhere, we shall describe Svp3p, an unrelated putative PtdIns(3,5)P_2 effector that influences PtdIns(3,5)P_2 metabolism and appears to contribute to MVB sorting and to heat tolerance (SK Dove, unpublished; see Figures 7B and D).

Other observations support the notion that PtdIns(3,5)P_2 exerts its effects through multiple effector pathways. For example, low overexpression of the *FAB1* gene in the

vac14-1 background restores vacuole acidification, but the vacuole remains enlarged. However, vacuole enlargement is only corrected if *FAB1* is greatly overexpressed (Bonangelino *et al*, 2002; Dove *et al*, 2002). Similarly, the catalytic site mutant *FAB1*$^{G2042V/G2045V}$ normalises MVB sorting in *fab1*Δ cells but does not correct the vacuole enlargement (Odorizzi *et al*, 1998).

Figure 8B summarises our current understanding of PtdIns(3,5)P_2 effector pathways. It highlights the fact that none of the currently known PtdIns(3,5)P_2 effectors mediates PtdIns(3,5)P_2-dependent vacuole acidification, and therefore suggests that at least one *S. cerevisiae* PtdIns(3,5)P_2 effector still awaits discovery. Furthermore, Hsv1p and Hsv2p are likely to be PtdIns(3,5)P_2 effectors, but their loss causes none of the known *FAB1*/PtdIns(3,5)P_2-dependent phenotypes—it is therefore probable that undiscovered PtdIns(3,5)P_2-dependent cellular processes remain to be found.

Materials and methods

Most materials were from sources defined previously, and methods for the growth of yeast, for FM4-64 vacuolar staining, for the GFP-Phm5p MVB sorting assay, for quinacrine staining and for lipid have been described (Dove *et al*, 1997, 2002; Dove and Michell, 1999; McEwen *et al*, 1999). The source of all yeast strains used in this study is indicated in Table I. PtdIns4*P* and PtdIns5*P* were from Echelon Inc. (Salt Lake City, UT), and PtdIns(3,4)P_2, PtdIns(4,5)P_2, PtdIns(3,4,5)P_3, and PtdIns3*P* were from Matreya Inc. (Pleasant Gap, PA): all were dipalmitoyl. Dioleoyl-PtdCho (DOPC), dipalmitoyl-PtdSer and PtdIns were from Sigma-Aldrich. Phosphoinositides and Affigel-linked PtdIns(3,5)P_2 were synthesised as described (Krugmann *et al*, 2002). For Biacore analysis, PtdIns(3,5)P_2 was from CellSignals Inc. (Lexington, KY).

Vacuole morphology screen

This screen was as described (Dove *et al*, 2002). Subsequent analysis of the identified genes included bioinformatic sifting to eliminate genes unrelated to vacuole function and to prioritise genes with homologues in many organisms.

Plasmid construction

GFP-Svp1p and GFP-Hsv2p were constructed by PCR amplification of the respective ORFs from yeast genomic DNA using the expand proof-reading DNA polymerase (Roche). The Met-regulated construct pUG36-SVP1 was created by ligating the *SVP1* ORF cut with *Eco*RI and *Hin*dIII into pUG36. pUG36-HSV2 was created by ligating the complete *HSV2* ORF, excised with *Bam*HI and *Sal*I, into pUG36. Transformed yeast were grown to 1×10^7 cells/ml in SC-Ura-Met and visualised as described (Dove *et al*, 2002). Mutations in the *SVP1* gene were carried out using serial overlap extension PCR (Warrens *et al*, 1997). Mutants were sequenced and then moved into GST expression vectors.

Expression of GST-Svp1p and GST-Hsv2p

The *SVP1* and *HSV2* ORFs were cloned into pGEX-6P-1 (Amersham-Pharmacia) (with a rhinovirus-2C protease cleavage site between GST and the target protein) and pGSTag (Ron and Dressler, 1992). Both plasmids were transformed into *Escherichia coli* BL21 and expressed as follows: cells were grown in LB + ampicillin (100 μg/ml) to an OD_{600} of 0.5–0.8, induced with IPTG (200–500 μM, 2–4 h, 30°C), harvested, washed in ice-cold PBS containing protease inhibitors (pepstatin A, 5 μg/ml; leupeptin 5 μg/ml; E-64, 3 μg/ml; aprotinin, 5 μg/ml; PMSF, 100 μM), lysed (100 mg/ml lysozyme, 4°C, 30 min) and disrupted with glass beads in a BeadBeater (6×30 s, 4°C). Triton X-100 (1%) supernatants from the disrupted cells (8000 *g*, 15 min; then 100 000 *g*, 1 h) were filtered (0.2 μm) and applied to glutathione–sepharose 4B beads (30 min, 4°C), which were eluted following the manufacturer's recommendations. Proteins were made 50% in ice-cold glycerol, snap-frozen and stored at −80°C.

Phosphoinositide dot blots

SVP1 and *HSV2* cloned into the pGSTag vector (with a PKA phosphorylation site in the GST linker) were expressed in *E. coli* BL21 (see above) and ^{32}P-labelled (Kavran *et al*, 1998). [^{32}P]proteins were filtered (0.2 μm) and used to probe lipid 'dot blots' of serial two-fold dilutions of phosphoinositides (from 1000 to 15.6 pmol, in $CHCl_3$:CH_3OH:H_20, 400:56:4, v/v/v) on Hybond C membrane (BioRad). Blots were blocked (3 h, room temperature, 10 ml PBS, 0.5 mM $MgCl_2$, 5% ECL blocking reagent) and probed with $1–2 \times 10^6$ dpm of [^{32}P]protein (30 min, room temperature). Blots were extensively washed (PBS/0.5 mM $MgCl_2$) and radioactivity detected by autoradiography. Concentrations of the lipid stocks were quantified immediately before spotting by wet ashing and phosphate determination (Baginski *et al*, 1967).

Protein binding to Affigel-linked PtdIns(3,5)P_2

GST-Svp1p (5 μM) was incubated (1 ml PBS containing 0.1% Triton X-100, 10 mM DTT and 50 mM PMSF) with 20 μl of Affigel-20 beads covalently linked to PtdIns(3,5)P_2, in the presence of micellar dispersions of various phosphoinositides (10 μM) for 30–60 min at 4°C. In 10 min or less, the beads were sedimented (12 000 *g*, 1 min) and washed in the above buffer (twice) and in 5 mM Hepes/KOH, pH 7.5. The remaining bead-bound protein was solubilised in 40 μl SDS–PAGE sample buffer and resolved on 10% SDS–PAGE minigels (made with piperazine diacrylamide (BioRad) rather than *N′N′*-methylene bis-acrylamide, to reduce the silver-staining background). Gels were fixed and stained (BioRad Silver Stain Plus).

Biacore analysis of phosphoinositide binding

This was carried out exactly as described (Yu and Lemmon, 2001). Positive controls were performed alongside Svp1p experiments, and included the PLCδ1 PH domain (binds PtdIns(4,5)P_2), the DAPP1 PH domain (binds PtdIns(3,4)P_2), the FAPP1 PH domain (binds PtdIns4*P*) and the Hrs1 FYVE domain (binds PtdIns3*P*). Lipid

Table I Yeast strains used in this study

Yeast strain	Source	Reference
BY4742 *mat* α *ura3*Δ*0 his3*Δ*1 leu2*Δ*0 lys2*Δ*0*	EUROSCARF	N/A
BY4742 *svp1*::*KANMX4*	EUROSCARF	N/A
BY4742 *svp1*::*KANMX4 hsv1*::*LEU2 hsv2*::*HIS3*	This study	N/A
BY4742 *hsv1*::*LEU2 hsv2*::*HIS3*	D Alexandraki	Georgakopoulos *et al* (2001)
BY4742 *hsv1*::*KANMX4*	EUROSCARF	N/A
BY4742 *hsv2*::*KANMX4*	EUROSCARF	N/A
BY4742 *fab1*::*KANMX4*	EUROSCARF	N/A
BY4742 *fab1*::*KANMX4 pho8*::*LEU2*	This study	N/A
BY4742 *hsv1*::*KANMX4 pho8*::*LEU2*	This study	N/A
BY4742 *hsv2*::*KANMX4 pho8*::*LEU2*	This study	N/A
BY4742 *svp1*::*KANMX4 pho8*::*LEU2*	This study	N/A
BY4742 *svp2*::*KANMX4*	EUROSCARF	N/A
BY4742 *svp3*::*KANMX4*	EUROSCARF	N/A

NA: not applicable.

surfaces were used within 8 h of generation, since signal strength began to decrease within ~12 h.

GST was quantitatively removed from GST-Svp1p with PreScission protease. When gel-filtered on Superose-6 (Amersham-Pharmacia), the liberated Svp1p eluted as a 60–70 kDa monomer. Svp1p binding was measured by simultaneously passing it over a phosphoinositide-containing sensor surface and a control DOPC sensor surface, with the control signal subtracted from that from the phosphoinositide surface. Binding data are plotted as per cent of maximum binding against protein concentration injected. K_D values were calculated as described (Yu and Lemmon, 2001).

Estimating K_D value

For Svp1p binding to PtdIns(3,5)P_2, PtdIns(3,4)P_2 and PtdIns(4,5)P_2, data were fitted to the following equation:

$$\text{Percent maximal binding} = \left(100 \frac{\left(\frac{[\text{Prot}]}{K_D}\right)}{\left(\frac{[\text{Prot}]}{K_D} + 1\right)} \right) + Y$$

where [Prot] is the flowing protein concentration (assumed unaffected by binding to the surface), K_D is the dissociation constant and Y corresponds to a residual or background signal. Fitting was performed using ORIGIN (MicroCal), with floating K_D and Y.

[^{3}H]inositol labelling and phosphoinositide analysis

Yeast were labelled in inositol-free media for 5–6 cell divisions, so that changes in [^{3}H] would parallel changes in lipid concentration, and phosphoinositides were extracted and analysed as described (Cooke *et al*, 1998).

Assay of Pho8Δ60p maturation

This used a method described by Noda *et al* (1995). *PHO8* was disrupted in appropriate strains using a *pho8*::*LEU2* cassette, and the disruptants were transformed with a plasmid that overexpresses Pho8Δ60p (pTN3, a gift from Dr T Noda, National Institute for Basic Biology, Okazaki, Japan). These strains were grown to ~1×10^7 cells/ml, washed and either processed immediately or incubated in a nitrogen-free medium for 8 h at 30°C, to induce autophagy and then processed. They were suspended in 1 ml of 50 mM Tris–HCl, 5 mM $MgCl_2$, 1 mM PMSF and 1 μg/ml pepstatin A, pH 9, cooled (15 min) and vortexed with glass beads (8×30 s, with 30 s on ice between cycles). Lysates were centrifuged (10 000 *g*, 15 min, 4°C). The supernatant protein (5 μg) was incubated at 25°C for 3 min in 1 ml of 10 mM *p*-nitrophenolphosphate, 0.5 M Tris and 2.5 mM $MgCl_2$, pH 8.8, with continuous monitoring of OD_{420}. Relative rates of dephosphorylation rates were calculated for the induced and uninduced cells.

Dilution assays for temperature sensitivity of growth

Yeast were diluted to 1×10^7 cells/ml, serially diluted four-fold, and 5 μl samples spotted on replicate plates. One was incubated at 23°C and the other at 42°C, until wild-type cells formed distinct colonies at all dilutions.

Assay for retrograde vacuole to late endosome trafficking

Cells expressing RS-ALP were spheroplasted as previously described (Urbanowski and Piper, 2001) and lysed in 300 mM sorbitol, 20 mM HEPES, pH 7.2, and 1 mM EDTA (HES buffer) containing a protease inhibitor cocktail (Complete tm, Boehringer Mannheim). Postnuclear supernatants were adjusted to 30% Optiprep and layered beneath 5 ml of HES buffer in an SW41 Beckman ultracentrifuge tube. Linear Optiprep gradients were overlaid (BioComp gradient mixer) and the samples were centrifuged (40 000 rpm, 18 h). Fractions were collected downwards from the top. Proteins were separated by SDS–PAGE and immunoblotted using monoclonal anti-ALP and anti-Vph1p antibodies (Molecular Probes) and a polyclonal anti-Vps10p antiserum (Piper *et al*, 1995).

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