

***Schizosaccharomyces pombe* Vps34p, a phosphatidylinositol-specific PI 3-kinase essential for normal cell growth and vacuole morphology**

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SUMMARY

We have cloned the gene, *vps34*⁺, from the fission yeast *Schizosaccharomyces pombe* which encodes an 801 amino acid protein with phosphatidylinositol 3-kinase activity. The *S. pombe* Vps34 protein shares 43% amino acid sequence identity with the *Saccharomyces cerevisiae* Vps34 protein and 28% identity with the p110 catalytic subunit of the mammalian phosphatidylinositol 3-kinase. When the *vps34*⁺ gene is disrupted, *S. pombe* strains are temperature-sensitive for growth and the mutant cells contain enlarged vacuoles. Furthermore, while wild-type strains exhibit substantial levels of phosphatidylinositol 3-kinase activity, this activity is not detected in the *vps34*Δ strain. *S. pombe* Vps34p-specific antiserum detects a single protein in cells of ~90 kDa that fractionates almost exclusively with the crude membrane fraction. Phosphatidylinositol 3-kinase activity also is localized mainly in the membrane fraction of wild-type cells. Immunoisolated Vps34p specifically

phosphorylates phosphatidylinositol on the D-3 position of the inositol ring to yield phosphatidylinositol(3)phosphate, but does not utilize phosphatidylinositol(4)phosphate or phosphatidylinositol(4,5)bisphosphate as substrates. In addition, when compared to the mammalian p110 phosphatidylinositol 3-kinase, *S. pombe* Vps34p is relatively insensitive to the inhibitors wortmannin and LY294002. Together, these results indicate that *S. pombe* Vps34 is more similar to the phosphatidylinositol-specific 3-kinase, Vps34p from *S. cerevisiae*, and is distinct from the p110/p85 and G protein-coupled phosphatidylinositol 3-kinases from mammalian cells. These data are discussed in relation to the possible role of Vps34p in vesicle-mediated protein sorting to the *S. pombe* vacuole.

Key words: PtdIns 3-kinase, vacuole, *Schizosaccharomyces pombe*

INTRODUCTION

Multiple mammalian phosphoinositide 3-kinases (PI 3-kinases) recently have been identified including the heterodimeric p110/p85 (Carpenter et al., 1990; Hiles et al., 1992; Panayotou et al., 1992) and a G-protein coupled PI 3-kinase (Stephens et al., 1994). The p110/p85 heterodimer is composed of a regulatory subunit (p85) and a catalytic subunit (p110). Multiple isoforms of the p110 and p85 subunits have been purified, cloned and sequenced (Gout et al., 1993; Hu et al., 1993; Hu and Schlessinger, 1994; Otsu et al., 1991). The p85 subunit, which lacks PI 3-kinase activity, mediates the interaction of the p110/p85 complex with activated growth factor receptor tyrosine kinases (Otsu et al., 1991). This is accomplished by sequence specific binding of the C-terminal SH2 domains of p85 to phosphorylated tyrosine residues in the activated receptors (Carpenter et al., 1993), and results in activation of the PI 3-kinase. Another portion of the p85 protein facilitates its interaction with p110 (Dhand et al., 1994). Analysis of purified p110 indicates that it has intrinsic PI 3-kinase activity (Hiles et al., 1992). The enzyme can phosphorylate phosphatidylinositol (PtdIns), phosphatidylinositol(4)phosphate

(PtdIns(4)P), and phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5)P₂) in an in vitro assay; however, the p110/p85 complex appears to have a marked preference for PtdIns(4,5)P₂ in vivo (Auger et al., 1989; Hawkins, 1992; Stephens et al., 1991). This suggests that a primary role for the receptor-associated PI 3-kinase is to produce phosphatidylinositol(3,4,5)-trisphosphate (PtdIns(3,4,5)P₃). Enzyme inhibitor and mutant studies have indicated that the p110/p85 PI 3-kinase regulates an array of cellular processes including: cell proliferation (Cantley et al., 1991), oxidative burst (Segal and Abo, 1993), membrane ruffling (Wennström et al., 1994), glucose uptake (Okada et al., 1994), and growth factor receptor endocytosis and recycling (Joly et al., 1994; Kapeller et al., 1993). However, the precise role that PI 3-kinase and 3-phosphorylated phosphoinositides play in these pathways remains unclear.

An additional mammalian phosphoinositide 3-kinase has recently been partially purified (Stephens et al., 1994) which is biochemically more similar to the yeast Vps34 enzyme than to the p110 or G-protein coupled enzyme (Stephens et al., 1994). Like Vps34p, this mammalian enzyme is a PtdIns-specific phosphoinositide 3-kinase (PtdIns 3-kinase) and is relatively wortmannin insensitive. This PtdIns 3-kinase is also

immunologically distinct from p110/p85, and is not activated by phosphotyrosine peptide treatment. Presently, the sequence of this protein is unknown, but it exhibits properties in common with the yeast Vps34p PtdIns 3-kinase, suggesting that it may also function in regulating protein sorting in the secretory pathway.

In *S. cerevisiae*, Vps34p function is required for protein delivery to the vacuole (Herman and Emr, 1990), which is the organelle analogous to the lysosome in mammalian cells. The vacuole contains a variety of hydrolytic enzymes and it is a major site of macromolecular turnover in the cell (Klionsky et al., 1990). Proteins destined for the vacuole transit through the early stages of the secretory pathway together with proteins destined to be secreted from the cells. In a late Golgi compartment, vacuolar hydrolases are sorted away from other secretory proteins and packaged into transport vesicles that deliver these enzymes to the vacuole via an endosomal intermediate compartment. Genetic selections have resulted in the identification of numerous *S. cerevisiae* mutants that exhibit defects in vacuolar protein sorting (Bankaitis et al., 1986; Jones, 1977; Robinson et al., 1988; Rothman and Stevens, 1986; Rothman et al., 1989).

The product of the *S. cerevisiae* *VPS34* gene shares substantial sequence identity with p110 (Schu et al., 1993) as well as other subsequently identified phosphoinositide kinases (Flanagan et al., 1993; Yoshida et al., 1994). Strains deleted for the *VPS34* gene or containing point mutations in *VPS34* are devoid of PtdIns 3-kinase activity, display a temperature sensitive growth defect, and mis-sort vacuolar hydrolases (Herman and Emr, 1990; Schu et al., 1993). Evidence indicating that Vps34p plays a direct role in regulating an early step in vacuolar protein sorting has been provided by a recently characterized *vps34^{ts}* mutant (Stack et al., 1995). This mutant expresses nearly normal levels of PtdIns 3-kinase activity at 25°C and vacuolar proteins are sorted properly to the vacuole. However, upon shifting these cells to 37°C, the kinase activity is rapidly inactivated and newly synthesized vacuolar hydrolases like carboxypeptidase Y are mis-sorted and secreted from the cells. Collectively, these data suggest that phosphoinositides and more specifically, PtdIns(3)P, play an essential role in the vesicle-mediated sorting and delivery of vacuolar enzymes.

To address whether *S. pombe* contains a Vps34-related PtdIns 3-kinase or p110-like PI 3-kinase we employed a PCR-based cloning strategy and isolated an *S. pombe* gene (*vps34⁺*, designated *Spvps34⁺* in this paper to emphasize the distinction from the *S. cerevisiae* *VPS34* gene) that encodes a phosphatidylinositol-specific PI 3-kinase. We report here that the *S. pombe* Vps34 PtdIns 3-kinase exhibits biochemical properties similar to *S. cerevisiae* Vps34p, and SpVps34p may be required for normal vacuole function.

MATERIALS AND METHODS

Strains, media, and yeast genetic methods

Escherichia coli strain XL1-Blue (Stratagene, La Jolla, CA) was used for all cloning procedures. The wild-type *S. pombe* strain used is called SP1 (*h⁺ leu1-32 ura4-D18*). Standard *S. pombe* (Moreno, 1991) and *E. coli* (Miller, 1972) media were used and supplemented as needed. *S. pombe* cells were transformed by the lithium acetate

method as described (Okazaki, 1990). The strain KTP1 is SP1 with the *vps34⁺* gene disrupted (*S. pombe vps34Δ*) and is described below.

Reagents

DNA restriction and modifying enzymes were obtained from Boehringer Mannheim or New England Biolabs. Zymolyase-100T was obtained from the Seikagaku Kogyo Co. (Tokyo, Japan) and protease-free novozyme was a kind gift from Dr Steen Mortensen. PtdIns, PtdIns(4)P, PtdIns(4,5)P₂, and phosphatidylserine were from Sigma. 5(6)-Carboxy-2',7'-dichlorofluorescein diacetate (CDCFDA) was from Molecular Probes, Inc. (Eugene, OR). Expre³⁵S protein labeling mix and [³H]myo-inositol were obtained from NEN Radiochemicals, and [α-³²P]dCTP, [α-³⁵S]dATP and [γ-³²P]ATP were from Amersham Laboratories. Unless indicated, all other chemicals were obtained from Sigma.

Plasmid constructions

Conventional recombinant DNA methods were used in the construction and propagation of all plasmids. The yeast shuttle vectors pRS426 (Christianson et al., 1992) and SEYC68 (Herman and Emr, 1990) have been described previously. The vector pART1 (McLeod, 1987), contains the *S. pombe* constitutive *adh⁺* promoter adjacent to the multiple cloning site, and the *S. cerevisiae* *LEU2* gene which serves as a selectable marker because it complements an *S. pombe* *leu⁻* strain (SP1, *leu1-32*).

PCR amplification and cloning of the *vps34⁺* gene

To amplify PI 3-kinase-like sequences from cDNA libraries of *S. pombe*, the following oligonucleotides were synthesized: (1) 5'GTTTGGATCC(A/G)(T/C)(A/T/G/C)AC(A/G)CA(A/G)TA(A/T/G/C)CC(A/T/G/C)GC(A/G)CA3'; (2) 5'GTTTCTGCAGG(A/T/G/C)GA(T/C)GA(T/C)(T/C)T(A/T/G/C)(A/C)G(A/T/G/C)CA(A/G)G A3'; (3) 5'GTTTGGATCC(A/T)(A/G)(A/T/G/C)CC(A/G)AA(A/G)TC(A/T/G/C)(A/G)(T/C)(A/G)TG3'. To facilitate cloning of the PCR products after amplification, *Bam*HI and *Pst*I restriction sites were incorporated at the 5' ends of the oligonucleotides. Oligonucleotide (1) encodes amino acids CAGYCV; (2) encodes GDDLRLQ; and (3) encodes H(I/A)DFG. The polymerase chain reaction (PCR) (Saiki, 1988) contained 2 μg *S. pombe* genomic DNA, 0.5 μg primers, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgSO₄, 0.2 mM dNTPs, and 5 units AmpliTaq™ DNA polymerase (Perkin Elmer-Cetus) in a total volume of 50 μl. The thermocycler was programmed to complete 30 cycles with the following protocol: 94°C for 1 minute, 51°C for 1 minute 30 seconds, 72°C for 2 minutes. The reaction product was digested with *Bam*HI and *Pst*I, and resolved by electrophoresis in a 1.5% agarose gel. The product was eluted from the gel and ligated into a pBluescript KS(-) acceptor vector that had been digested with *Bam*HI and *Pst*I. The cloned PCR fragment was sequenced using the Sequenase kit (US Biochemicals).

Following confirmation that a PI 3-kinase-like gene fragment had been amplified, the entire *vps34⁺* gene was isolated by probing a pUC9-based *S. pombe* cDNA library (a gift from P. Nurse) with the PCR fragment. The cloned PCR product was labeled with [α-³²P]dCTP by random primer synthesis (Feinberg and Vogelstein, 1983) and purified using a Sephadex G-50 column. Then, it was hybridized to nitrocellulose filter replicas of bacterial colonies containing the *S. pombe* cDNA library. One positive cDNA clone was obtained and subcloned into pBluescript to generate a restriction map and create deletions for sequencing.

Disruption of the *vps34⁺* gene

The *vps34⁺* locus was disrupted in the SP1 wild-type *S. pombe* strain by replacing an internal *vps34⁺* gene fragment with the *S. pombe* *ura4⁺* gene to produce strain KTP1. A 0.8 kb *Xba*I-*Xba*I fragment was eliminated from the cloned *vps34⁺* open reading frame and a 1.6 kb *ura4⁺* cassette (Grimm et al., 1988) was inserted. A linearized DNA fragment carrying this disrupted *vps34⁺* gene was used to

transform a wild-type haploid strain. In order to confirm that the *vps34⁺* gene had been disrupted, PCR analysis was performed by a previously described technique (Herman and Emr, 1990). *S. pombe* cells were lysed with glass beads, nucleic acid was extracted with phenol/chloroform, and an aliquot of the nucleic acid was used for the PCR analysis. Each DNA template was mixed with a set of three 25 base oligonucleotide primers that hybridize to specific regions of either the *vps34⁺* gene or *ura4⁺* locus.

Overexpression of *S. pombe* Vps34p

For the purpose of expressing *vps34⁺* under the control of a strong promoter, the 2.6 kb *EcoRI* cDNA fragment containing *vps34⁺* was inserted into pRS426. The pRS426-*vps34⁺* construct was digested with *SalI* and *BamHI*, and this fragment was ligated into pSEY68 (Herman and Emr, 1990). The *vps34⁺*-containing *PstI* fragment of pSEY68-*vps34⁺* was ligated into pART1 (McLeod, 1987). The final plasmid, pKTP1, contains the *S. cerevisiae* *LEU2* gene as a selectable marker (complements an *S. pombe* *leu⁻* mutation) and the *vps34⁺* gene behind the *S. pombe* *adh* promoter. This plasmid was transformed into strain KTP1 (*h⁺ leu1-32 ura4-D18, vps34Δ*, see above) to yield KTP1/pKTP1.

Fluorescent labeling of cells and microscopy

S. pombe cells were labeled with CDCFDA at 30°C as previously described (Alfa et al., 1993). Cells were grown to mid-log phase, harvested by centrifugation and resuspended in low pH YE medium prior to staining. For staining, 5 μl of 1 mM CDCFDA was added to 1 ml of cells and the cells were incubated at 30°C for 0.5 hour with constant rocking. The stained cells were pelleted, washed, and resuspended in 0.1 ml of low pH YE medium. Cells were affixed to glass slides, then examined and photographed using a Nikon Microphot-SA microscope equipped with Nomarski and fluorescence optics.

Preparation of *S. pombe* Vps34p-specific antisera

A fusion between the *E. coli* *trpE* gene and the *vps34⁺* gene was constructed by subcloning a 0.54 kb *XbaI/HindIII* fragment encoding amino acids 507-687 of SpVps34p into *XbaI/HindIII* digested pATH2 (Dieckman and Tzagoloff, 1985), generating an in-frame fusion gene. Induction of the TrpE-SpVps34 fusion protein was accomplished as previously described by Kleid et al. (1981). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) revealed a prominent 55 kDa band representing the fusion protein which was excised and electroeluted from the gel slices using a Schleicher & Schuell Electro-Separation chamber. Approximately 500 μg of the purified fusion protein was emulsified with Freund's complete adjuvant and injected intramuscularly and subcutaneously into a young male New Zealand white rabbit. Antiserum was collected and screened by immunoprecipitation.

Cell labeling and immunoprecipitation of the *S. pombe* Vps34 protein

For analysis of SpVps34p, *S. pombe* cells were grown to mid-log phase in MB medium (Moreno et al., 1991) at 30°C with appropriate amino acids. Cells were pelleted by centrifugation (2,000 g for 5 minutes) and resuspended in 1 ml of MB medium containing 1 mg/ml bovine serum albumin and 250 μCi of Expres³⁵S-label. The cells were labeled at 30°C for 20 minutes and then chased at 30°C for 40 minutes with excess unlabeled methionine (5 mM) and cysteine (1 mM). The label/chase reactions were terminated by the addition of trichloroacetic acid to a final concentration of 6%. Cells were pelleted and washed with acetone twice before lysis. Cells were lysed by vortexing in the presence of glass beads and protein was solubilized by incubating in a denaturing buffer (50 mM Tris-HCl, pH 7.5, 6 M urea, 1% SDS) at 70°C for 5 minutes. Lysates were cleared by centrifugation, the supernatant diluted ten-fold into immunoprecipitation buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% Tween-20, 0.1 mM EDTA), anti-Vps34p antiserum was added, and this solution incubated at 4°C for 12-16 hours with constant rocking. Protein A-

Sepharose (Pharmacia) was added to the solution for 1.5 hours in order to collect the antigen:antibody complexes. The Protein A-Sepharose/antigen:antibody complexes were pelleted by centrifugation, washed twice with immunoprecipitation buffer and twice with this solution containing 2 M urea. Then, antibody and immunoprecipitated protein were dissociated by incubating in SDS-PAGE solubilization buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 2% β-mercaptoethanol, 6 M urea) at 70°C for 5 minutes. The labeled, immunoprecipitated protein was analyzed by SDS-PAGE/fluorography as previously described (Klionsky et al., 1988), or signal from ³⁵S-labeled protein was quantitated using a Phosphorimager (Molecular Dynamics).

HPLC analysis of in vivo phosphoinositides

S. pombe cellular phosphoinositides were labeled with 10 μCi/ml [³H]myo-inositol (specific activity=18.8 Ci/mmol; Amersham) by growing cells in minimal medium lacking inositol. Cellular lipids were extracted and PI(3)P and PI(4)P levels were analysed using high performance liquid chromatography (HPLC) by a previously described method (Stack et al., 1995).

Enzyme assays and cellular fractionations

Cell extracts for the in vitro PtdIns 3-kinase assays were prepared as described previously (Stack et al., 1993). For the fractionation experiments, *S. pombe* cells were grown in MB medium, spheroplasted, and resuspended in 0.1 M KCl, 15 mM Hepes, pH 7.5, 3 mM EGTA and 10% glycerol at a concentration of 15-20 A₆₀₀ units/ml. The cells were lysed by vortexing in the presence of glass beads and protease inhibitors. The lysates were cleared by centrifugation at 1,000 g for 10 minutes, and the resulting supernatant was centrifuged at 100,000 g for 30 minutes at 4°C to generate P100 and S100 fractions. The pellet was resuspended in a volume equal to the supernatant and aliquots of the supernatant and pellet were used in the in vitro PtdIns 3-kinase assay. For the whole cell extracts and fractionated samples, the 50 μl reactions were performed in 20 mM Hepes, pH 7.5, 10 mM MgCl₂, 0.2 mg/ml sonicated PtdIns, 60 μM ATP and 0.2 mCi/ml [^γ-³²P]ATP. Reactions were carried out at 25°C for 5 minutes and terminated with the addition of 80 μl of 1 M HCl. Lipids were extracted with 160 μl of chloroform-methanol (1:1) and the organic phase was dried and stored at -80°C. The samples were resuspended in chloroform and spotted onto CDTA-treated Silica Gel 60 thin layer chromatography (TLC) plates (Merck) which were then developed using a borate solvent system (Walsh et al., 1991). The ³²P-labeled lipids were detected by autoradiography or signal was quantitated with a Phosphorimager.

Native immunoprecipitation and substrate specificity of *S. pombe* Vps34p

The native immunoprecipitation technique was performed essentially as described (Stack et al., 1993). Immunoprecipitated Vps34p was used directly in an in vitro PtdIns 3-kinase assay with PtdIns as the substrate (see above for details) or in a phosphoinositide kinase assay with different substrates. For the substrate specificity experiments, the phosphoinositide kinase assays were done the same as above except for the following changes: phosphatidylserine was added as carrier (0.2 mg/ml) and either PtdIns (PI), PtdIns(4)P (PIP), or PtdIns(4,5)P₂ was provided as substrate (0.2 mg/ml) in the reaction. The reactions were performed and lipids extracted as described above. The reaction products were separated on potassium oxalate-treated Silica Gel 60 plates developed with a 1-propanol/2 M acetic acid (65:35) solvent system (Auger et al., 1992). Unlabeled standards were visualized by staining with iodine vapors.

Wortmannin and LY294002 inhibition of *S. pombe* Vps34p PI 3-kinase activity

For this analysis, in vitro PtdIns 3-kinase assays were performed (see above) using the wild-type *S. pombe* strain, SP1, in the presence of

wortmannin (Sigma) or LY294002 (a kind gift from C. Vlahos, Lilly Research Labs). Inhibitor and lysates were added to a pre-mixed solution containing buffer, MgCl₂, and substrate (PtdIns) and incubated at 25°C for 5 minutes prior to initiating the reaction with ATP. Standard in vitro PtdIns 3-kinase reactions were carried out and products were separated on Silica Gel 60 TLC plates. Signal from the labeled products was quantitated using a Phosphorimager.

RESULTS

Cloning of the *S. pombe vps34⁺* gene

Degenerate oligonucleotides encoding the most highly conserved sequences within the catalytic domain of *S. cerevisiae* Vps34p and bovine p110 were used to PCR amplify a 300 bp gene fragment from *S. pombe* genomic DNA (see Materials and Methods). The PCR product encoded an open reading frame that had significant sequence identity with *S. cerevisiae* Vps34p. This gene fragment was used as a probe to recover the *vps34⁺* gene (the *S. pombe vps34⁺* gene will be referred to as *Spvps34⁺* to clearly distinguish it from the *S. cerevisiae VPS34* gene) from an *S. pombe* cDNA library. One positive cDNA clone was isolated and sequenced. The nucleotide sequence of the cDNA clone contained a single large open reading frame predicted to encode a protein of 801 amino acids (Fig. 1A). Alignment of the deduced amino acid sequence of *Spvps34⁺* with the sequences from *S. cerevisiae* Vps34p and bovine p110 showed that the *S. pombe* protein was most similar to Vps34p (43% overall identity). Moreover, the degree of sequence identity was highest over the C-terminal catalytic domains of these two proteins (60% identity, Fig. 1B). The homology with mammalian p110 (28% overall identity) was restricted to the C-terminal catalytic domain of the protein (Fig. 1B).

Sequence comparison of the catalytic domains of Vps34p, SpVps34p, and p110 reveals motifs which are highly conserved in both protein kinases and other PI kinases. Included are motifs which have been implicated via crystal structure analysis to have a role in ATP binding and phosphate transfer in cAMP-dependent protein kinase (Knighton et al., 1991). The catalytic loop region which has a consensus DXHXXN (residues 657 to 662 of SpVps34p, X is any amino acid) and the DFG (residues 675 to 677) motif which is the smallest and most highly conserved structural feature of protein kinases are conserved in these phospholipid kinases. Furthermore, there are additional domains which are conserved in the phosphoinositide kinases that vary among the protein kinases. These patches of sequence identity may have a role in phosphoinositide kinase activity; they may participate in substrate (phospholipids) binding or in the transfer of phosphate to lipids. The most striking of these domains are located in the SpVps34p protein at residues 545 to 556 (FKN...DQL) and 637 to 656 (DNY...GVG). Altogether, this comparison illustrates the significant sequence conservation between all three proteins in their C-terminal domains, and the overall sequence identity between the *S. pombe* and *S. cerevisiae* Vps34 proteins suggesting they may be functional homologs.

Spvps34⁺ disruption affects cell growth and vacuole morphology

To examine the phenotypic consequences of a null allele of

Spvps34⁺, we performed a gene deletion-disruption of this locus. A linear fragment of the *Spvps34⁺* gene in which the 0.8 kb *XbaI-XbaI* internal fragment had been replaced with the *S. pombe ura4⁺* gene (Fig. 2A) was used to transform SP1 (*h⁺ leu1-32 ura4-D18*) haploid cells. Consistent with the observation that the *VPS34* gene is not essential in *S. cerevisiae*, many candidate *ura4⁺*-disrupted *Spvps34⁺* transformants were obtained. Several *ura⁺* transformants were isolated and the structure of the disrupted allele was verified by southern blot analysis or by PCR amplification of the locus (data not shown). We then examined the growth phenotype and cellular morphology of the strain, KTP1, in which *Spvps34⁺* was disrupted.

Colonies of wild-type (SP1) and *Spvps34Δ* (KTP1) cells were streaked onto YPD plates and incubated at 26°C or 36°C for 4 days. While both wild-type and *Spvps34Δ* *S. pombe* cells grew at 26°C, *Spvps34Δ* cells exhibited a temperature-sensitive growth defect at 36°C (Fig. 2B). This result is similar to what is observed in *S. cerevisiae Δvps34* strains (Herman and Emr, 1990); disruption of the *VPS34* gene renders cells unable to survive at 37°C, which may result from decreased tolerance to temperature stress due to abnormal vacuolar function. Microscopic examination of the cells revealed vacuolar morphological defects associated with loss of *Spvps34⁺* function (Fig. 3). A fluorescent dye that accumulates in the vacuole (CDCFDA) was used to visualize the vacuoles in wild-type and *Spvps34Δ* mutant cells. Wild-type *S. pombe* cells contain numerous small vacuoles (Fig. 3). These fragmented vacuoles appeared to be randomly dispersed throughout the cells. In contrast, *Spvps34Δ* cells had one to three large vacuoles which were detected both by CDCFDA staining and in Nomarski images. These enlarged vacuoles did not segregate properly to both daughter cells during division. All of the observed growth and morphological defects seen in *Spvps34Δ* cells were complemented after introduction of the wild-type *Spvps34⁺* gene on the expression plasmid pKTP1 (see Materials and Methods). Together, these experiments demonstrate that loss of SpVps34p function in the *Spvps34Δ* strain had a significant impact on the growth and vacuolar morphology of these cells.

Spvps34Δ mutant lacks PtdIns 3-kinase activity

Vps34p is a PtdIns-specific 3-kinase and when *S. cerevisiae* strains are deleted for *VPS34*, the cells lack detectable PtdIns(3)P, but PtdIns(4)P levels remain high (Schu et al., 1993; Stack et al., 1993, 1995). We began the characterization of SpVps34p activity by examining levels of in vivo phosphoinositides in wild-type and *Spvps34Δ* strains (Fig. 4). *S. pombe* cells were labeled with [³H]inositol, lipids were extracted, deacylated and glycerophosphoinositol head groups analyzed by anion-exchange HPLC. Data obtained from the HPLC

Fig. 1. SpVps34p is homologous to *S. cerevisiae* Vps34p and bovine p110. (A) The nucleotide sequence and deduced amino acid sequence of *vps34⁺* (referred to as *Spvps34⁺*) are illustrated. Numbering of amino acids starts with the first methionine of the open reading frame. (B) Alignment of a C-terminal portion of the SpVps34 amino acid sequence with the most conserved sequences of *S. cerevisiae* Vps34p and bovine p110. The residues which are conserved between the different phosphoinositide 3-kinases are shown in the black boxes. Spaces were introduced into all three sequences and residues were omitted from the p110 (indicated in the parenthesis) sequence in order to maximize alignment. Amino acid numbering is indicated at the beginning and end of each sequence.

A.

1 GAATTCCTGTATGGTAAAAAGTGAATTTCCAAAAATAAATGCTTTACATATGGA 56
 57 ATGGATAGACTTGTTTTTTCATATTGCTCTTCAAGCAAGGTGACTGCCAGATCTTGGTTAAATTTGCTTTCATTTGAATATCAAGATAGTCAAGAACCATGCATTTGCACCATTCAATG 176
 1 M D R L V F S Y C P S S K V T A R F L V K F C F I E Y Q D S Q E P C I C T I Q L 40
 177 TTTTCAGGGAATGAATCAGGATCTTTAATGCAAAAATGTTTCGGTTTCAAAAATACCAATAAGAGTCTACTTCTACTGAGCTTTCTAAGATATCAACTCATGAGTGGCTCGACTTTGGG 296
 41 F S G N E S G S L M Q K C F V S K I P N K S L L P T E L S K I S T H E W L D F G 80
 297 GTTACCCTTCTGAGCTCTCATTGAACGCAAAAATTTGTAGTGTCTGCTGCAAAACCATCTTAAATGATGAGGAAGTCTATGAATTTGTGGGCTGTACAACGTATCGACTTTTGTAGAA 416
 81 V T V S E L S L N A K F V V S A W K P S F N D E E V Y E F V G C T T Y R L F D E 120
 417 AATAATTTACTAAGGCAAGGTCTCCAAAAATACCTTTACAGACTTCAAAAAGAAATAAAGAACTACTCGCAACATCGTTGGAAC TAGAACAAAGTTAAAGAAAATAAACCGGCTTGACGGT 536
 121 N N L L R Q G L Q K I P L Q T S K E I K K Y S P T S L E L E Q V K E I N R L D G 160
 537 TTACTTCTACAATACAGTTGGGCGATGTTCCATCCGTTAATTTGGCTGGATGATATATCCTTTGGAAAAATCAAGACTTTAGATCTAAGCACATGTCTCTCGTCACTATCCCTATTTTG 656
 161 L L L Q L Q L G D V P S V N W L D D I S F G K I K D F R S K H M S L V T I P I L 200
 627 TATCTTGTATTTCTCAATTTCTCTTTCCCGGCTGCTTTCAACGATCCTACTTCCCAATCTGAAAATCGAGTATATTTACTCGTCTTTGATTTGGAGTTAAATCTAGATAGTCCAGT 776
 201 Y L D F L Q F S F P V V F Q R S Y Y P K S E N R V Y Y S S F D L E L N L D S P A 240
 777 GAGCTGAACATAGACGACTGGTTCGTTCTCAACGTAATGGACCTTTGGATAAGGACTTAAACCTAATTTCAAAAATTCGAAAAGAGCTTGAGTCAATTTTATCTTATCCCAAGCGAA 896
 241 E L K H R R L V R S Q R N G P L D K D L K P N S K I R K E L E S I L S Y P P S E 280
 897 GAATTATCACTTGAAGAAAAGGACCTTATTTGGAAGTTTAGATTTTACTTACCAGGAATAAGAAAAGCAATGACTAAATTTGAAAAGCGTGTGTTGCACTGATTCATCGAAGTCAAT 1016
 281 E L S L E E K D L I W K F R F Y L T R N K K A M T K F L K S V V W T D S S E V N 320
 1017 CAAGCACTAGTCTTTAGATAGCTGGACCGCAAAATGACATGACGATGCAATGGAATTTACTAAGTCTGATTTGTACACCTAAGGTTGCTGCTACGCTGTCTCTCGTTLTAGAGAT 1136
 321 Q A L S L L D S L E I D I D D A L E L L S P S F V H P K V R A Y A V S R L L E T 360
 1137 GCTTCGAATGAAGAATTTGTTGTTATACCTTCTCCAACCTGTTCAAGCATTACGATATGACAAACCTATCAGCTCTGATGAAAGGTTCCAACCTAGCCTCTTCCCTTATTTCTGTAAT 1256
 361 A S N E E L L L Y L L Q L V Q A L R Y D N P I S S D E R F Q P S P L A L F L V N 400
 1257 AGAGCAATATCATCCGAGTATCGGAAATGATTTATTTGGTACTTAGTAGTAGAGATGAAGATGAGCCCGTAAGCAAGCTATTTTCTCCGCTCATGTTCTTTTCAAAAAGAGCTT 1376
 411 R A I S S P I S G N D L Y W Y L V E I E D E P V S K L F S S V M F L F Q K E L 440
 1377 TCAAAGTCTGGAAGGAAGATGATCCGCGAAACACTTTCTGCTCAGGCAAAATCGTTGAAAATTTACTACGCATAAGTAAATCTGTACAATCATTTCCGGTACTCGATTGAAGAAA 1496
 441 S K S V E G R L I R E T L S A Q A K F V E K L L R I S K S V Q S F R G T R L K K 480
 1497 ATTGAATCTTAAAAGTCTTTTGAAGATCATAAGTATCTTACTGGATTTTCTGCTTTACCCTTCTCTAGATCCATCCGTAATATAGTTGGCATTATCCAGATGCCTGTAGC 1616
 481 I E Y L K V L L E D H K Y H L L D F H A L P L P L D P S V N I V G I I P D A C T 520
 1617 GTATTCAATCATACCATGCACCTTTACGCTTACTTTTTAAATGCCAAGATGGATCAAATATCCTATAATTTAAAACGGTGATGATTACGTAAGACAGCTTGTATTCAAATA 1736
 521 V F K S T M Q P L R L L F K C Q D G S K Y P I I F K N G D D L R Q D Q L V I Q I 560
 1737 TTAACACTTATGGATAAGCTGTTGAAGAAAAGAAAATTTGATTTGATTTAAAGCCCTATAGAATCCTTGCAACGGGTCCTCACTCATGGTCCGCTACAATTTGTACCGCTAAAACCGTT 1856
 561 L T L M D K L L K K E K L D L H L K P Y R I L A T G P T H G A V Q F V P S K T L 600
 1857 GCTACTATTTTAGCAGAATATCATGTAAGTGTGTTTAGCTTATTTACGAGAAAACCAATCCAGATGATGGAATTTCTGCGAATACGGGATAGACCTGTTGCTATGGACAACTACGTT 1976
 601 A T I L A E Y H G S V L A Y L R E N N P D D G L N S A N Y G I D P V A M D N Y V 640
 1977 CGAAGCTCGCAGGTTATTTGTGAATCACTTATTTATAGGAGTTGGTGTATCGGCCTTAGACAATCTCTTGATTACGAAAGACGGTCATTTTTCATGCTGACTTTGGTTATATTTCTG 2096
 641 R S C A G Y C V I T Y L L G V G D R H L D N L L A I T K D G H F F H A D F G Y I L 680
 2097 GGTCGAGATCCTAAGCTTTTCCACGACTATGAACCTTTCTAAGGAAATGGTTGAAGGTATGGGGGCTACAATTTCCCTTTTATCAGCAATTTAAATCTTATGCTATACAACCTTT 2216
 681 G R D P K L F S P A M K L S K E M V E G M G G Y N S P F Y Q F K S Y C Y T T F 720
 2217 ACTGCTTACGAAAATCTCGAACCTGATCCTTAACTTTGATGATGGTTGATGCGAATATACCAGATATAAAATTTGACAAGAAAAGTAGTATATAAGTCAAAGAAAAGATT 2336
 721 T A L R K S S N L I N L F S L M V D A N I P D I K F D K E K V V Y K V K E R F 760
 2337 TGTTTCAAAATGCTGAATCAGATGCCATCAAATTTTGAACAATTAATTAATGATTTGTGTCAGTGTCTGTTTCTCAAAATCATAGATCGCATGCACAATTTGCGCAGATATATGCGA 2456
 761 C L Q M S E S D A I K Y F R Q L I N D S V S A L F P Q I I D R M H N L A Q Y M R 800
 2457 TCCTAA 2462
 801 S * 801
 2463 ACATTAATGACTTAAATGATTATATGCCACATTTACACTTTTTTTTTTATCCAAACTTGCTTTTCCATTATAGCTGTGTTGAACTAATACATTATTATGCCTAGTAAAAAGGTTGTGTT 2583
 2584 TTCTAAAGAAAGTGATGAATTTGATAATGACGGTGTGTAATTTACCGAATT 2635

B.

SpVps34 541- **Y P I I F K N G D D L R Q D Q L V I Q I L T L M D K L L K K E K L D L H L K P Y R I L A T G P T H G**
 ScVps34 619- **Y H L M F K V G D D L R Q D Q L V V Q I I S L M N E L L K N E N V D L K L T P Y K I L A T G P Q E G**
 p110 797- **N E I I F K N G D D L R Q D M L T L Q I I R I M E N I W Q N Q G L D L R M L P Y G C L S I G D C V G**

SpVps34 **A V Q F V P S K T L A T I L A E Y H G S V L A Y L R E N N P D D G L N S A N Y G I D P V A M D N Y V R S C**
 ScVps34 **A I E F I P N D T L A S I L S K Y H G - I L G Y L K L H Y P D E - - - N A T L G V Q G W V L D N F V K S C**
 p110 **L I E V V R N - - S H T I (17aa) H T L H Q W L K D K - - N K G E I Y D A A I - - - - - D L F T R S C**

SpVps34 **A G Y C V I T Y L L G V G D R H L D N L L I T K D G H F F H A D F G Y I L G R D P K L F S P A M K L S K -**
 ScVps34 **A G Y C V I T Y I L G V G D R H L D N L L V T P D G H F F H A D F G Y I L G Q D P K P F P P L M K L P P Q**
 p110 **A G Y C V A T F I L G I G D R H N S N I M V K D D G Q L F H I D F G H F L D H K K K K F (8aa) F V L (5aa)**

SpVps34 **M V E G M G G Y N S P F Y Q Q F K S Y C Y T T F T A L R K S S N L I L N L F S L M V D A N I P D I K F D K**
 ScVps34 **I I E A F G G A E S N Y D K F R S Y C F V A Y S I L R R N A G L I L N L F E L M K T S N I P D I R I D P**
 p110 **I V I S K G A Q E C (6aa) E R F Q E M C K A Y L A - I R Q H A N L F I N L F S M M L G S G M P E - - L Q S**

SpVps34 **E K V V Y K V K E R F C L Q M S E S D A I K Y F E Q L I N D S V S A L F P Q I I D R M H N L A Q Y M R S 801**
 ScVps34 **N G A I L R V R E R F N L N M S E E D A T V H F Q N L I N D S V N A L L P I V I D H L H N L A Q Y W R T 875**
 p110 **F D D I A Y I R K T L A L D K T E Q E - A L E Y F M (5aa) A H H G W T T K M D W I F H T I K Q H A L N 1068**

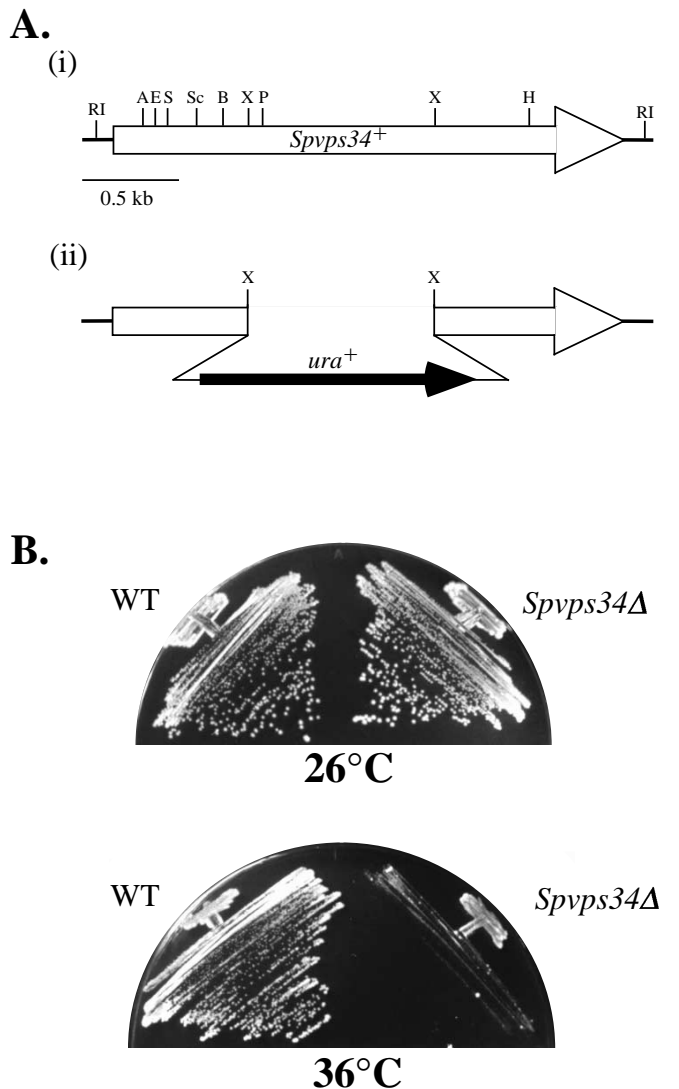


Fig. 2. Disruption of *Spvps34⁺* and characterization of strain growth. (A, i) A restriction map of the *Spvps34⁺* gene is shown with the open reading frame indicated by an open arrow. Restriction sites are abbreviated as follows: RI, *EcoRI*; A, *AccI*; E, *EcoRV*; S, *SacI*; Sc, *ScaI*; B, *BglII*; X, *XbaI*; P, *PvuII*; H, *HindIII*. (ii) The 0.8 kb *XbaI-XbaI* fragment within the open reading frame of *Spvps34⁺* was replaced by the *S. pombe ura4⁺* gene (black arrow) to generate the deletion/disruption strain, KTP1. (B) The growth phenotypes of wild-type and *Spvps34Δ* cells on YPD plates at 26°C and 36°C were examined. Plates were incubated at the indicated temperature for 4 days.

analysis showed that wild-type cells contain significant PtdIns(3)P and PtdIns(4)P, represented by the levels of gPI(3)P and gPI(4)P, respectively. The ratio of PtdIns(3)P to PtdIns(4)P was approximately 0.5 in *S. pombe* compared to approximately 1.3 in *S. cerevisiae* (Stack et al., 1995). In contrast to wild-type, no PtdIns(3)P was detected in the *Spvps34Δ* strain, but the *S. pombe Spvps34Δ* strain appeared to maintain wild-type levels of PtdIns(4)P. Therefore, disrupting *Spvps34⁺* in these cells eliminated their capacity to synthesize PtdIns(3)P and suggests that *Spvps34⁺* encodes an active PtdIns 3-kinase.

PtdIns 3-kinase activity was also measured in cell extracts from wild-type and *Spvps34Δ* cells. Extracts were incubated

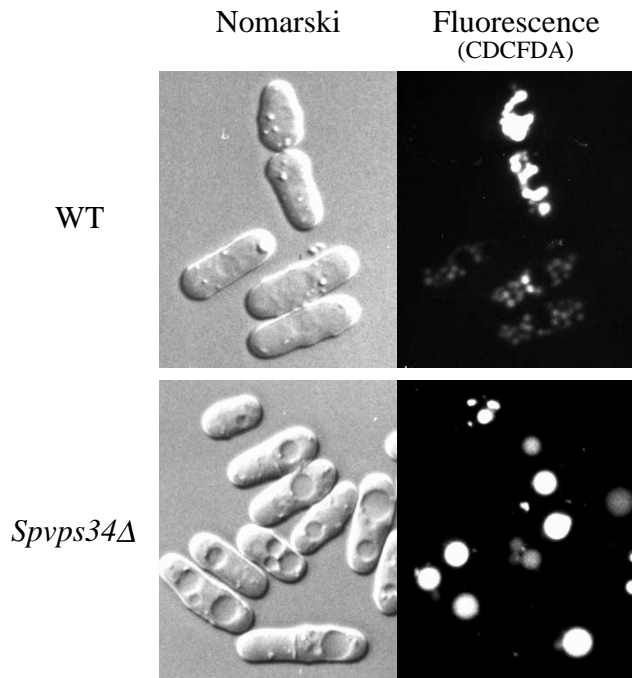


Fig. 3. Vacuolar morphology in wild-type and *Spvps34* mutant cells. Wild-type (WT) and *Spvps34Δ* cells were grown in YPD at 26°C to mid-log phase and stained with CDCFDA by the method described in Materials and Methods. The stained cells were then visualized using Nomarski optics (left panels) and fluorescence microscopy (right panels).

with PtdIns and [γ - 32 P]ATP in the PtdIns 3-kinase assays. The lipid products from the assays were extracted and analyzed by thin layer chromatography. There was significant PtdIns 3-kinase activity in the wild-type strain and no detectable PtdIns 3-kinase activity in *Spvps34Δ* cells (Fig. 5), while both strains exhibited equivalent PtdIns 4-kinase activity. We also examined the PtdIns 3-kinase activity found in extracts of cells where *Spvps34⁺* is expressed under the control of the strong, *adh⁺* promoter (*Spvps34Δ/pKTP1*, strain KTP1/pKTP1). Multiple experiments using the strain *Spvps34Δ/pKTP1*, indicate that PtdIns 3-kinase activity (Fig. 5) was approximately 10-15-fold higher than that found in wild-type cells. These data confirmed the analysis of in vivo PtdIns(3)P levels, and extended those findings by showing that enhanced expression of *Spvps34⁺* resulted in increased cellular PtdIns 3-kinase activity.

SpVps34 PtdIns 3-kinase activity associates with the membrane fraction

Since the production of PtdIns(3)P in *S. pombe* cells was a result of SpVps34p activity, we examined whether SpVps34p was associated with a membrane or cytosolic fraction by following the fractionation pattern of the PtdIns 3-kinase activity (Fig. 6). This was a first step in defining the subcellular site of action of the SpVps34 PtdIns 3-kinase, and provided information concerning the role of this enzyme in vivo. For this analysis, cleared *S. pombe* cellular lysates were centrifuged at 100,000g to yield supernatant (S100) and pellet (P100) fractions, and phosphoinositide kinase activity in these fractions was assessed using

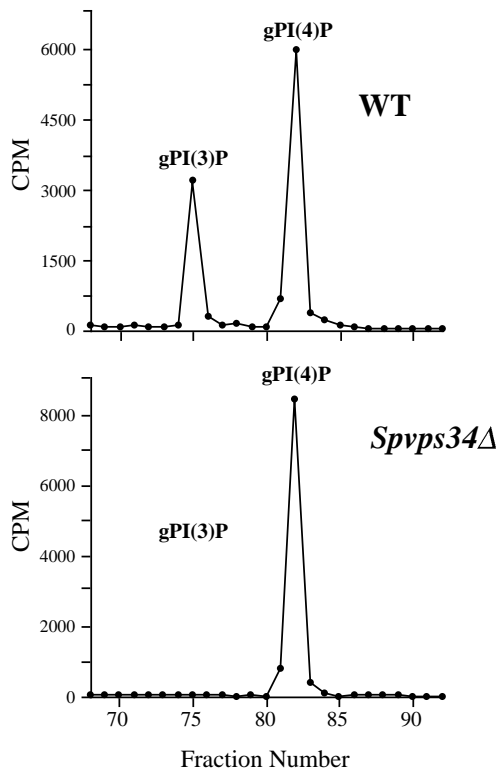


Fig. 4. Analysis of in vivo levels of PtdIns(3)P and PtdIns(4)P in wild-type and *Spvps34Δ* strains. Wild-type and *Spvps34Δ* strains were grown for 20 hours at 30°C in medium containing [³H]myo-inositol. Extraction and deacylation of cellular lipids was performed as detailed in Materials and Methods. HPLC analysis of ³H-labeled glycerophosphoinositols was accomplished using a strong anion-exchange column and glycerophosphoinositols were eluted with an ascending gradient of (NH₄)₂PO₄. Fractions (20 seconds each) were collected and counts per minute (cpm) determined with a liquid scintillation counter. Glycerophosphoinositide(3)phosphate (gPI(3)P) and glycerophosphoinositide(4)phosphate (gPI(4)P) peaks were identified by comparing their elution position in the gradient to the elution times of previously run ³²P standards.

the in vitro PtdIns 3-kinase assay. In wild-type cells, the PtdIns 3-kinase activity was found exclusively in the P100 membrane fraction, and the PtdIns 4-kinase activity was localized primarily to the P100 with some activity being found in the S100 fraction. In the strain deleted for the *Spvps34⁺* gene, there was no detectable PtdIns 3-kinase activity in either the P100 or S100 fraction, while the PtdIns 4-kinase activity fractionated in a manner indistinguishable from wild type. In addition, we tested to see if the PtdIns 3-kinase fractionation pattern was altered in the strain KTP1/pKTP1 (*Spvps34Δ/pKTP1*). When *Spvps34⁺* was expressed under the control of the *adh⁺* promoter, the pattern of localization was altered. There was now detectable PtdIns 3-kinase activity in the S100 fraction, although, the majority of the PtdIns 3-kinase activity was still associated with the P100 fraction.

SpVps34p is a PtdIns-specific phosphoinositide 3-kinase

To further characterize the SpVps34 protein, we prepared antiserum against a TrpE:SpVps34p fusion protein. In

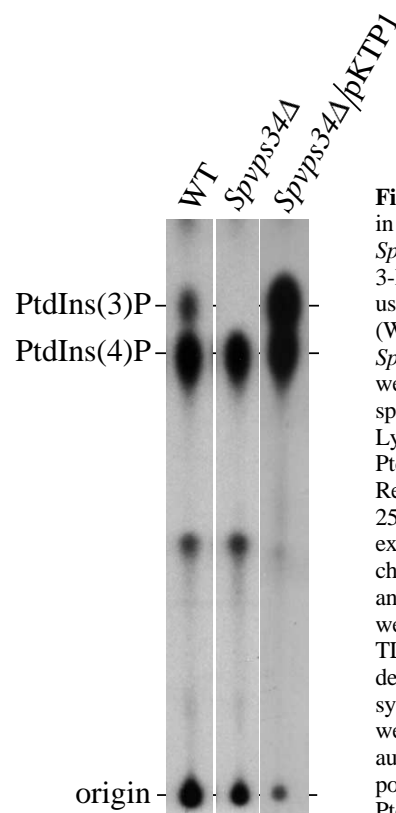


Fig. 5. PtdIns 3-kinase activity in wild-type, *Spvps34Δ*, and *Spvps34Δ/pKTP1* cells. PtdIns 3-kinase assays were performed using extracts from wild-type (WT), *Spvps34Δ*, and *Spvps34Δ/pKTP1* strains. Cells were grown to mid-log phase, spheroplasted, and lysed. Lysates were then added to the PtdIns 3-kinase reactions. Reactions were conducted at 25°C for 5 minutes, lipids were extracted with chloroform/methanol and analyzed by TLC. Samples were spotted onto Silica Gel 60 TLC plates which were developed with a borate solvent system. ³²P-labeled products were detected by autoradiography. The migration positions for PtdIns(3)P and PtdIns(4)P are indicated.

immunoprecipitation experiments, the antiserum detected a single protein which had a size (~90 kDa) matching the predicted molecular mass of SpVps34p. This protein was observed in extracts from wild-type, but not *Spvps34Δ* cells (Fig. 7A). Several experiments indicated that strain KTP1/pKTP1 (*Spvps34Δ/pKTP1*) contained 7-10-fold higher levels of SpVps34 protein than the wild-type strain. We employed the SpVps34p-specific antiserum to immunoaffinity purify SpVps34p from KTP1/pKTP1 cells. The native immunoprecipitation technique yielded samples highly enriched in SpVps34p PtdIns 3-kinase activity which were essentially free from contaminating PtdIns 4-kinase activity (Fig. 7B, lane 2, compare native IP to whole cell). The purified SpVps34p was added to the standard in vitro PtdIns 3-kinase reactions; however, instead of using only PtdIns (PI) as the substrate, PtdIns(4)P (PIP) and PtdIns(4,5)P₂ (PIP₂) were tested as substrates. The products from these independent reactions were extracted and analyzed by TLC and autoradiography. SpVps34p acts solely on PtdIns (PI) to produce PIP (Fig. 7C, PI lane) and not on PIP or PIP₂ (Fig. 7C, PIP and PIP₂ lanes); no higher phosphorylated forms of PtdIns (PIP₂ or PIP₃) are produced in these reactions. The data in these experiments clearly demonstrated that SpVps34p utilized PtdIns exclusively as a substrate to produce PtdIns(3)P. We conclude that SpVps34p is a PtdIns-specific 3-kinase, which places it in a class with Vps34p and mammalian PtdIns-specific phosphoinositide 3-kinases.

Wortmannin and LY294002 inhibit SpVps34p PtdIns 3-kinase activity

The fungal metabolite wortmannin inhibits mammalian PI 3-

kinase apparently by binding irreversibly to the p110 catalytic subunit of PI 3-kinase (Thelen et al., 1994; Yano et al., 1993). This compound has been used to address the role of PI 3-kinase

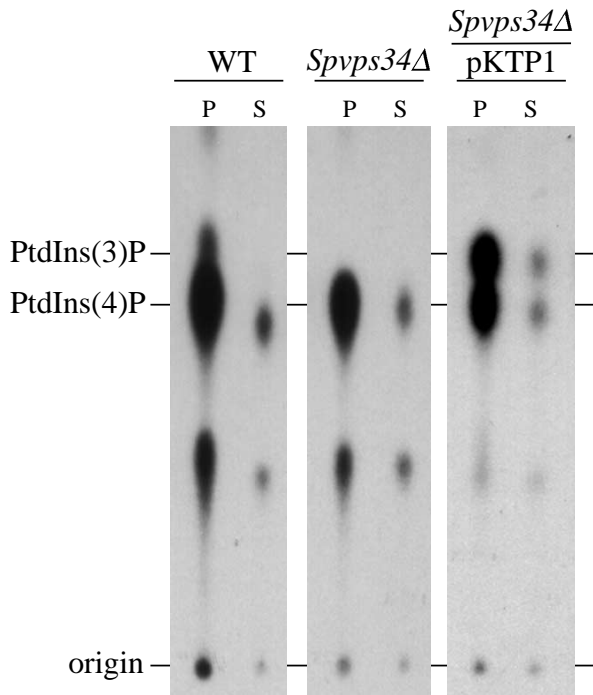
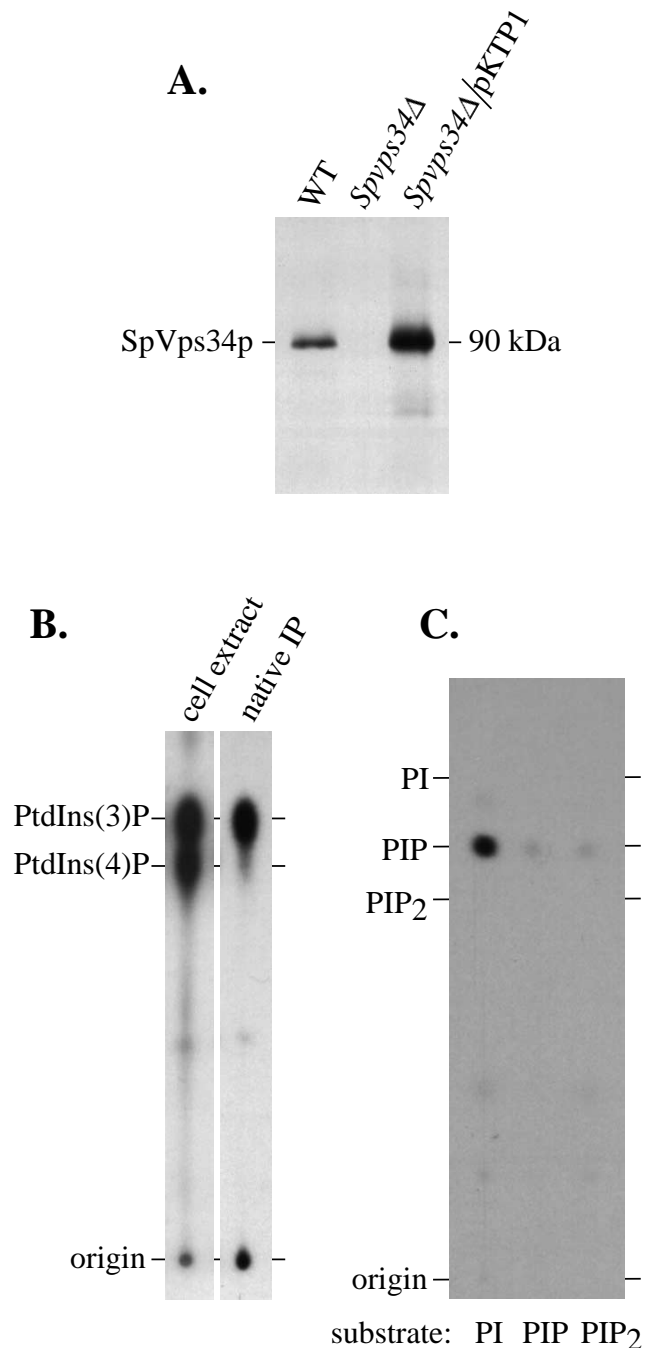


Fig. 6. SpVps34p PtdIns 3-kinase activity associates with the particulate cell fraction. *S. pombe* cell lysates were fractionated by ultracentrifugation as described in Materials and Methods and P100 (P) and S100 (S) fractions were assayed for PtdIns 3-kinase activity as in Fig. 5. Labeled lipids from wild-type, *Spvps34Δ*, and *Spvps34Δ/pKTP1* strains were extracted with chloroform/methanol and analyzed by TLC. Samples were spotted onto silica gel 60 plates which were developed with a borate solvent system. The ^{32}P -labeled products were detected by autoradiography. The migration positions of PtdIns(3)P and PtdIns(4)P are indicated.

Fig. 7. SpVps34 is a PtdIns-specific phosphoinositide 3-kinase. (A) Levels of SpVps34p in wild-type, *Spvps34Δ*, and *Spvps34Δ/pKTP1* cells were determined by immunoprecipitation with anti-SpVps34p antiserum as described in Materials and Methods. Immunoprecipitate from 5 A_{600} units of cells was electrophoresed in each lane. ^{35}S -labeled proteins were detected by SDS-PAGE/fluorography. (B) PtdIns 3-kinase assays were performed using extracts (whole cell) or native immunoprecipitated SpVps34p (native IP) from a strain in which *Spvps34⁺* is expressed under the control of the *S. pombe adh⁺* promoter (*Spvps34Δ/pKTP1*). Reaction products were analyzed by TLC on silica gel 60 plates developed with a borate solvent system, and ^{32}P -labeled lipids were detected by autoradiography. The migration positions of PtdIns(3)P and PtdIns(4)P are indicated. (C) For the substrate specificity assays, SpVps34p was obtained by native immunoprecipitation from strain *Spvps34Δ/pKTP1*. Phosphoinositide kinase assays were performed at 25°C for 5 minutes in the presence of phosphatidylserine carrier (0.2 $\mu\text{g}/\mu\text{l}$). ^{32}P -labeled lipids were extracted with chloroform/methanol and analyzed on potassium oxalate-treated Silica Gel 60 plates developed with a propanol/acetic acid solvent system. Labeled lipids were detected by autoradiography. To identify the positions of PI, PIP, and PIP₂, unlabeled standards were run in an adjacent lane and stained with iodine vapors.

activity in signaling a variety of cellular responses. The potency and specificity of wortmannin in whole cells or in vitro make it a powerful diagnostic tool. The calculated IC₅₀ (half-maximal inhibition) of wortmannin for p110/p85 PI 3-kinase is in the range of 1-5 nM for several different mammalian cell types (Okada et al., 1994; Thelen et al., 1994; Yano et al., 1993), while it has no apparent effect below 100 nM on PtdIns 4-kinase activity. For the G-protein coupled PI 3-kinase, activity the IC₅₀ is 43 nM, but the mammalian PtdIns-specific phosphoinositide 3-kinase is relatively insensitive to wortmannin with an IC₅₀ greater than 100 nM (Stephens et al., 1994). This data correlates well with what was observed for the yeast PtdIns-specific 3-kinases, Vps34p and SpVps34p.



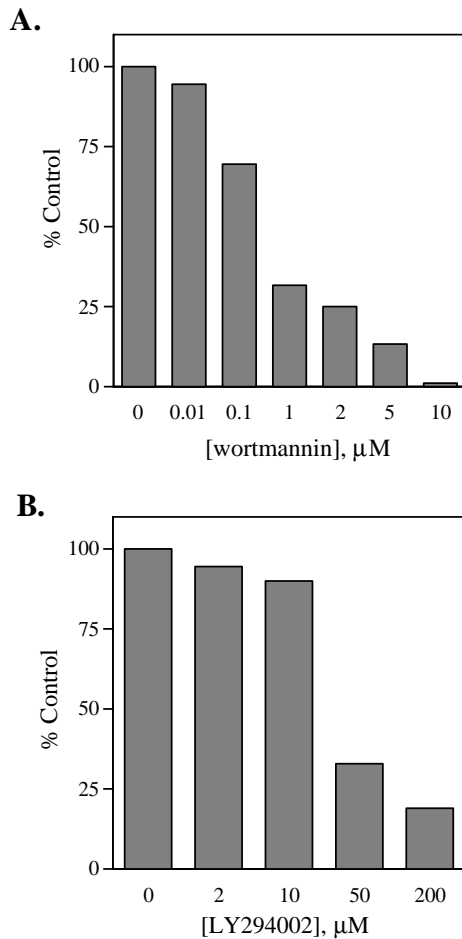


Fig. 8. Wortmannin and LY294002 inhibit the PtdIns 3-kinase activity of SpVps34p. Extracts from wild-type *S. pombe* were incubated in wortmannin (A) or LY294002 (B) for 5 minutes prior to performing PtdIns 3-kinase assays as described in Fig. 5. Quantitation of ^{32}P incorporated into PtdIns(3)P was accomplished using a Phosphorimager. The control PtdIns 3-kinase reactions have no inhibitor added. % Control refers to the cpm incorporated into PtdIns(3)P in a reaction where inhibitor was added, calculated as the percentage of the uninhibited reaction. The data presented are the average of two experiments.

The IC_{50} of wortmannin for Vps34p is approximately 3 μM (Stack and Emr, 1994), and we have determined that it is approximately 600 nM for SpVps34p (Fig. 8A). In addition, we confirmed earlier reports stating that wortmannin was highly specific for PI 3-kinase activity, and was much less effective on PtdIns 4-kinase activity (Yano et al., 1993). We observed no significant inhibition of PtdIns 4-kinase activity when wortmannin concentrations were below 5 μM (data not shown).

To extend our characterization, we also examined the effect of the compound LY294002 on SpVps34p PtdIns 3-kinase activity. LY294002 is an analog of quercetin, and is a specific and reversible inhibitor of mammalian p110/p85 PI 3-kinase (IC_{50} = 1.4 μM) (Vlahos et al., 1994.) We examined the effect of LY294002 on PtdIns 3-kinase activity in extracts from wild-type *S. pombe* and found that SpVps34p was also relatively insensitive to this compound with an IC_{50} of approximately 35

μM . Altogether, these data indicate that SpVps34p PtdIns 3-kinase activity is somewhat more sensitive to wortmannin and LY294002 than Vps34p, but SpVps34p is not significantly affected by concentrations of these compounds which completely inhibit the p110/p85 mammalian PI 3-kinase.

DISCUSSION

In an effort to determine whether the requirement for PtdIns 3-kinase activity in vacuolar protein sorting in *S. cerevisiae* is conserved among other eukaryotes, we cloned a gene from *S. pombe* that encodes a PtdIns 3-kinase. SpVps34p is a hydrophilic protein of 801 amino acids with a C-terminal catalytic domain that shares significant sequence identity with phosphoinositide kinases from *S. cerevisiae* (Vps34p; 43% identity, Herman and Emr, 1990), plant (SPI3K; 42% identity, Hong and Verma, 1994; AtVps34p; 42% identity, Welters et al., 1994), and mammalian cells (p110; 28%, Hiles et al., 1992). The SpVps34 protein has PtdIns 3-kinase activity and the cellular levels of PtdIns 3-kinase activity increase when SpVps34p is overexpressed. *S. pombe* haploid cells deleted for the *Spvps34*⁺ gene display multiple defects including: temperature sensitive growth, abnormal vacuolar morphology, and no detectable PtdIns 3-kinase activity. The SpVps34 PtdIns 3-kinase activity associates primarily with a membrane fraction. In addition, the SpVps34 PtdIns 3-kinase specifically phosphorylates PtdIns to yield PtdIns(3)P, and is relatively insensitive to the PI 3-kinase inhibitors, wortmannin and LY294002. These data are consistent with observations made in *S. cerevisiae* and suggest that SpVps34p, like *S. cerevisiae* Vps34p, may have a role in vacuolar protein sorting (see Table 1).

The mammalian p110 catalytic subunit of PI 3-kinase shares sequence identity with *S. cerevisiae* Vps34p (Hiles et al., 1992; Schu et al., 1993) and initially it was proposed that these enzymes may have a common cellular function. In fact, mammalian PI 3-kinase does have a role in growth factor receptor endocytosis and targeting to the lysosome (Joly et al., 1994; Kapeller et al., 1993), but additional analysis showing disparate substrate specificities suggest the mammalian enzyme may regulate a broader set of functions than the yeast enzyme. The p110/p85 PI 3-kinase has the capacity to phosphorylate PtdIns, PtdIns(4)P, and PtdIns(4,5)P₂. However, growth factor stimulation of PI 3-kinase activity results in formation of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃, while PtdIns(3)P levels remain constant (Stephens et al., 1993). This suggests that PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ may have distinct functions from PtdIns(3)P. At present, the precise functions of these more highly phosphorylated forms of PtdIns remain unclear, but they have been associated with a variety of cellular responses including: cell proliferation (Cantley et al., 1991), membrane ruffling in fibroblasts (Wennström et al., 1994), oxidative burst in neutrophils (Okada et al., 1994; Segal and Abo, 1993; Thelen et al., 1994), and in glucose transport control in adipocytes (Okada et al., 1994). In contrast to mammalian cells, PtdIns(3,4,5)P₃ has not been detected in yeast, and the only identified phosphoinositide 3-kinase in *S. cerevisiae* is Vps34p and it is PtdIns-specific. Characterization of *S. cerevisiae* Vps34p indicates that it plays an essential role in vacuolar protein sorting and that PtdIns(3)P may serve as a signaling molecule that triggers vesicle-mediated sorting of

Table 1. Properties of SpVps34p, Vps34p and p110

	SpVps34p	Vps34p	p110
Molecular mass	90 kDa	100 kDa	110 kDa
Substrate specificity	PtdIns	PtdIns	PtdIns, PtdIns(4)P PtdIns(4,5)P ₂
Wortmannin IC ₅₀	600 nM	3 μM	1-5 nM
Protein kinase activity	None detected*	Autophosphorylation	Autophosphorylation, phosphorylates p85 [†] and IRS-1 [‡]
Hetero-oligomeric partner	Unknown	Vps15p	p85
Regulated cellular process	Vacuolar morphology [§] cell growth	Vacuolar protein sorting	Membrane ruffling, oxidative burst, PDGF-receptor endocytosis/degradation, glucose uptake

* Protein kinase assays were performed by a method used to detect Vps34p autophosphorylation (Stack and Emr, 1994) and phosphorylation of SpVps34p was not detected.

[§] Possibly protein sorting events.

[†] Dhand et al., 1994.

[‡] Lam et al., 1994.

vacuolar hydrolases (Herman and Emr, 1990; Schu et al., 1993; Stack et al., 1993, 1995). We are currently examining the role of SpVps34p in signaling events related to cell growth and vesicle transport.

SpVps34p shares several properties in common with *S. cerevisiae* Vps34p, which implies that these proteins are functional homologs. We have expressed the *S. cerevisiae* VPS34 gene in the *Spvps34Δ* strain and found that this strain grew better at 36°C (data not shown), but still not as well as a wild-type strain. Also, similar to *S. cerevisiae* Vps34 PI 3-kinase activity, SpVps34p PtdIns 3-kinase activity associates with the membrane fraction of cell extracts. Hydrophathy analysis (data not shown) indicates that SpVps34p is relatively hydrophilic and lacks any apparent transmembrane domains. In *S. cerevisiae*, Vps34p associates with the membrane as a member of a heterodimeric complex with Vps15p, a serine/threonine protein kinase which is also required for vacuolar protein sorting. Vps15p recruits Vps34p to the membrane, and an active Vps15 protein kinase is required for the interaction (Stack et al., 1995). It is likely that *S. pombe* Vps34p also associates with a Vps15p-like molecule. Efforts are underway to identify this molecule.

There are presently no well-characterized vacuolar marker proteins in *S. pombe* like carboxypeptidase Y in *S. cerevisiae*. The vacuolar abnormalities observed in *Spvps34Δ* cells indicate that organelle function has been compromised, however, at present it is not possible to directly test if the observed morphology and growth defects result from missorting of vacuolar proteins. We have observed that total carboxypeptidase activity is several-fold lower in *Spvps34Δ* cells compared to wild-type *S. pombe* cells (K.T., unpublished). Because carboxypeptidase Y activity is due to enzymes located in the vacuole of *S. cerevisiae*, by analogy this suggests that *S. pombe Spvps34Δ* cells missort carboxypeptidase Y. Ultimately, a specific *S. pombe* vacuolar marker protein will need to be identified so that its sorting can be directly analyzed. Further study should illuminate whether the *S. pombe* PtdIns

3-kinase is a member of a family of eukaryotic PI 3-kinases whose primary role is to facilitate vesicle-mediated protein sorting.

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While this manuscript was being reviewed, Volinia et al. (1995) reported the cloning of a gene encoding a human Vps34p PtdIns PI 3-kinase homologue that exhibits biochemical properties similar to the yeast Vps34 proteins.