Saccharomyces cerevisiae contains a Type II phosphoinositide 4-kinase

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The yeast *Saccharomyces cerevisiae* contains two known phosphoinositide 4-kinases (PI 4-kinases), which are encoded by *PIK1* and *STT4*; both are essential. *Pik1p* is important for exocytic transport from the Golgi, whereas *Stt4p* plays a role in cell-wall integrity and cytoskeletal rearrangements. In the present study, we report that cells have a third PI 4-kinase activity encoded by *LSB6*, a protein identified previously in a two-hybrid screen as interacting with *LAS17p*. Although *Pik1p* and *Stt4p* are closely related members of the Type III class of PI 4-kinases, *Lsb6p* belongs to the distinct Type II class, based on its amino acid sequence, its sensitivity to inhibition by adenosine and its insensitivity to wortmannin. *Lsb6p* is the first fungal Type II enzyme cloned. The protein was expressed and purified from Sf9

cells and used to define kinetic parameters. As commonly observed for surface-active enzymes, activities varied both with substrate concentration and lipid/detergent molar ratios. Maximal activities of approx. 100 min⁻¹ were obtained at the PI/Triton X-100 ratio of 1:5. The *K_m* value for ATP was 266 μM, intermediate between the values reported for mammalian Type II and III kinases. Epitope-tagged protein, expressed in yeast, was entirely particulate, and about half of it could be extracted with non-ionic detergent. *Lsb6p*–green fluorescent protein was found both on vacuolar membranes and on the plasma membrane, suggesting a role in endocytic or exocytic pathways.

Key words: *LSB6*, phosphoinositide, protein trafficking, yeast.

INTRODUCTION

Phosphoinositide phosphates (PIPs) participate in many important cellular processes, including protein trafficking, signal transduction and cytoskeletal rearrangements [1,2]. The first step in the major canonical pathway towards PIP synthesis is catalysed by the phosphoinositide 4-kinases (PI 4-kinases). These enzymes have been classified for many years as Type II or III forms based on enzymic properties [3]. (The Type I enzyme was found to be a PI 3-kinase.) The Type III enzymes are generally soluble and sensitive to wortmannin, whereas the Type II enzymes are particulate and insensitive to the inhibitor. Although the sequences of the Type III enzymes have been known for several years, the first cDNA coding for a Type II enzyme was determined only recently [4,5].

Saccharomyces cerevisiae contains two known PI 4-kinases, encoded by *PIK1* and *STT4* [6,7]. Both are in the Type III family and are essential genes. The pools of phosphoinositide 4-phosphate [PI(4)P] generated by the two kinases seem to be spatially segregated. *PIK1* genetically interacts with *SEC14* and is important for exocytic trafficking from the Golgi [8,9]. PI(4)P generated by *Pik1p* facilitates the binding of *Kes1p* to the Golgi, a factor that controls ARF (ADP-ribosylation factor) function and budding [10]. A temperature-sensitive mutant also exhibits defects in endocytosis and vacuolar dynamics [11]. In contrast, *Stt4p* binds to the plasma membrane via the protein *Sfk1p*, where it promotes cell-wall synthesis, actin cytoskeleton organization and the ρ -mediated mitogen-activated protein kinase cascade [11,12]. Overproduction of one of these kinases cannot compensate for a gene disruption in the other [11]. It has been hypothesized that only these two PI 4-kinases exist in yeast, since a strain conditionally defective in both *Pik1p* and *Stt4p* contains less than 5% of the wild-type levels of PI(4)P under restrictive conditions [11].

Analysis of the first Type II PI 4-kinase (PI4KII) sequence recently revealed potential orthologues throughout the plant and animal kingdoms and possibly even in bacteria [4]. One such gene exists in the yeast *S. cerevisiae*, namely *Las17p*-binding protein (*LSB6*). *LSB6* was identified in a two-hybrid screen by using *LAS17*, an organizer of the cortical actin network, as bait [13]. No other results regarding the function of *LSB6* have been presented. In the present study, we found that *LSB6* indeed encodes a PI 4-kinase. We report the enzymic properties of the enzyme and show that the epitope-tagged protein is particulate and largely limited to the plasma membrane and vacuolar membrane, suggesting a role in the endocytic or exocytic pathways.

MATERIALS AND METHODS

Yeast strains

MMYO11 α [14] was the host strain for these studies. A Δ *lsb6* strain in MMYO11 α was generated by replacing the open reading frame (ORF) with the *HIS3* gene. For this purpose, *HIS3* was amplified by PCR with primers that also contained 50 bases of sequence immediately upstream and downstream of the *LSB6*-coding region; homologous recombination at the *LSB6* site and replacement of the *LSB6* ORF with *HIS3* were verified by PCR.

Chemicals

FM4-64 was purchased from Molecular Probes (Eugene, OR, U.S.A.). Anti-[glyceraldehyde-3-phosphate dehydrogenase (GADPH)] antibody was from Sigma (St. Louis, MO, U.S.A.). Anti-haemagglutinin (HA) antibody (monoclonal 12CA5) from BABCO (now available at Roche Diagnostics, Indianapolis, IN, U.S.A.) was a gift from Melanie Cobb (Department

Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HA, haemagglutinin; *LSB6*, *Las17p*-binding protein; ORF, open reading frame; PI 4-kinase, phosphoinositide 4-kinase; PI4KII, PI 4-kinase Type II; PIP, phosphoinositide phosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PI(4)P, phosphoinositide 4-phosphate.

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of Pharmacology, University of Texas Southwestern Medical Center, Dallas, U.S.A.). Anti-myc antibody (monoclonal 9E10) was obtained from the National Cell Culture Center (Minneapolis, MN, U.S.A.). Anti-poly-His antibody was purchased from Clontech (Palo Alto, CA, U.S.A.). PCR amplification utilized VENT polymerase (New England Biolabs, Beverly, MA, U.S.A.), with oligonucleotides from Sigma-Genosys (The Woodlands, TX, U.S.A.). Restriction enzymes were obtained from New England Biolabs and BacPak6 from Clontech. Protein G-Sepharose was purchased from Zymed Laboratories (San Francisco, CA, U.S.A.) and Ni-nitrilotriacetic acid-agarose from Qiagen (Valencia, CA, U.S.A.). [³H]myo-Inositol was purchased from New England Nuclear (Boston, MA, U.S.A.) and PI from Avanti PolarLipids (Alabaster, AL, U.S.A.). A kit containing PI and PIP species used to determine enzyme specificity was purchased from Echelon Biosciences (Salt Lake City, UT, U.S.A.).

Plasmid constructions

Yeast vectors

For epitope tagging Lsb6p with HA and myc, the *LSB6* ORF was first amplified with *FseI* and *AseI* ends and inserted into either pCS2+MT [15] (sequence available at http://sitemaker.umich.edu/dlturner.vectors/files/cs2_plus_mt.txt) or pCS+HA (pCS2+ with the following *BamHI*–*EcoRI* fragment cloned into the polylinker I of the vector: GG ATC CCA TCG GGG ATG GAG CGC GAC CGC GGT GGC GGC CGC ATC TTT TAC CCA TAC GAT GTT CCT GAC TAT GCG GGC TAT CCC TAT GAC GTC CCG GAC TAT GCA GGA TCC GAT TCG AA); pCS+HA was a gift from HongTao Yu (UT Southwestern, Dallas, TX, U.S.A.). The two vectors were also digested with *FseI* and *AseI*. The resulting constructs place the *LSB6* ORF downstream of six copies of the myc epitope or two copies of the HA epitope respectively. Both epitope-tagged *LSB6*-coding sequences (myc- and HA-Lsb6p) were then amplified with *BamHI* ends. The parent vectors consisted of pRS316, a low-copy-number vector [16], into which was inserted the phosphoglycerate kinase promoter–terminator (from the vector pEMBLy3012; a gift from Michael White, UT Southwestern) at the *HindIII* site. The *LSB6* PCR products, digested with *BamHI*, were inserted into the *BglII* site (which separates the *PGK* promoter and terminator) of the vectors to generate pRS316-PGK-myc-Pik2p and pRS316-PGK-HA-Pik2p (*PIK2* was our original laboratory term for *LSB6* before it was so named).

In parallel constructions, myc-Lsb6p and tagless Lsb6p were cloned into the multi-copy vector pRS426 [17] to generate pRS-426-PGK-myc-Pik2p and pRS-426-PGK-Pik2p respectively.

To generate Lsb6p–green fluorescent protein (GFP), the *LSB6* ORF was amplified with *BamHI* and *SpeI* ends, and cloned into the D005 plasmid cut with *BglII* and *XbaI* to remove the Pmp47 fragment. D005 is identical with D004 [18] except that the *XbaI* site in the polylinker was destroyed by digesting pRS315+PGK with *XbaI*, generating a blunt end, and then re-ligating. The addition of the *LSB6* ORF into D005 results in a GFP fusion such that a threonine–arginine spacer resides between the last codon of Lsb6p and GFP. The resulting plasmid is termed pRS315-PGK-Pik2p-GFP.

Sf9 insect cell vector

LSB6 ORF was amplified from yeast genomic DNA using the primers BACpac-5 (AAACGCGGATCCATGCATCACCATCACCATCACGGAGGTAGTAACGAAGCTTACCAGCA) and BACpac3 (AAACTGCAGTCAACACCAGGTGAATA-

CGG). BACpac-5 contained sequences encoding a *BamHI* site, methionine, His₆ and the start of the ORF. BACpac3 contained the end of the ORF and a *PstI* site. The resulting PCR product was digested with *BamHI* and *PstI* and inserted into pBacPAK6 according to the manufacturer's instructions (Clontech), to generate Bac-hisPIK2 for expression in Sf9 cells. The sequence of this insert and all PCR products containing coding regions were verified.

Localization studies

Subcellular fractionation

Yeast cells containing pRS316-PGK-HA-Pik2p or empty vector were cultured on minimal dextrose (SD) plates with appropriate auxotrophic supplements. Exponential-phase cells were converted into spheroplasts and lysed osmotically [19]. Lysis was judged microscopically to be approx. 90% complete. The suspension was centrifuged sequentially at 550 g for 20 min (Beckman JA17), 2200 g or 150000 g for 20 min (Beckman TLA100.3) and equivalent amounts of supernatants or pellets were processed for immunodecoration and visualized by the ECL[®] system (Amersham, Piscataway, NJ, U.S.A.).

Microscopy

Yeast cells expressing Lsb6p–GFP were observed using a Zeiss Axiovert 100 inverted microscope equipped with a 100 W mercury vapour lamp, a ×63 magnification oil-immersion lens, appropriate filter sets and OpenLab software (Improvision, Lexington, MA, U.S.A.). The vacuolar membrane was visualized using the endocytosed vital stain FM4-64 [20]. To verify that there was no channel spillover, cells lacking GFP or FM4-64 were observed.

Preparation of Lsb6p for enzyme analysis

myc-Lsb6p from yeast extracts

For detection of activity from myc-Lsb6p expressed in yeast, 100A₆₀₀ units of cells (equivalent to a 100 ml culture at an absorbance of A₆₀₀ 1) containing pRS316-PGK-myc-Pik2p and growing in exponential phase in SD medium (Yeast Nitrogen Base; Difco, Detroit, MI, U.S.A.), 2% dextrose and appropriate amino acid and base supplements were washed twice in water and broken with glass beads in 1 ml of enzyme buffer [20 mM Tris/HCl (pH 7.5), 10% glycerol, 0.1 M NaCl, 1% (v/v) Triton X-100 and a range of protease inhibitors] [4]. Lysates were incubated for 30 min on ice with unliganded Protein G–Sepharose beads and spun in the microfuge for 5 s. Supernatants were then incubated with myc antibody, which had been chemically cross-linked to Protein G–Sepharose, for 16 h at 4 °C with rocking, washed three times in enzyme buffer, and finally resuspended in 100 µl of the same solution.

His₆-Lsb6p from insect cells

Baculovirus was produced using Bac-hisPIK2 and BacPAK6 DNA according to the manufacturer's instructions. Production of His-tagged Lsb6p was monitored by immunoblotting using monoclonal antibodies specific to the His tag. Amplified baculovirus was produced using standard methods [21]. Protein was produced by infecting Sf9 cells with amplified baculovirus at 27 °C in a shaking incubator. After 50 h of infection, cells were harvested and lysed on ice in 20 ml of Buffer A [20 mM Hepes (pH 8.0)/2 mM MgCl₂/100 mM NaCl/0.2 mM PMSF] containing 2% Triton X-100. A supernatant was obtained by centrifuging the lysate at 105000 g in a Ti45 rotor for 30 min at 4 °C. The His₆-Lsb6p protein was purified by exposing the lysate to

Ni-nitrilotriacetic acid-agarose, washing the column with Buffer A containing 0.1 % Triton X-100 and 0.03 M imidazole, and eluting the protein from the resin using Buffer A containing 0.1 % Triton X-100 and 0.15 M imidazole. Fractions containing the purified protein were pooled and dialysed to remove the imidazole, and aliquots were frozen at -70°C .

PI 4-kinase assay

Kinase activity of His-tagged Lsb6p purified from Sf9 cells or myc-tagged enzyme immunoprecipitated from yeast lysates was measured essentially by a method described in [4]. Briefly, PI in chloroform was dried under nitrogen, reconstituted in resuspension buffer [50 mM Tris/HCl (pH 7.0)/1 mM EGTA/0.5 mg/ml BSA/Triton X-100 (typically 0.4 %)] with sonication, and the resuspension was spun in a microfuge for 2 min to remove aggregates. Standard reaction mixtures of 48 μl containing 300 μM PI, 3 mM Triton X-100 (concentrations adjusted as indicated in Figure 4), 1.6 μg of purified enzyme or 25 μl of resuspended myc-Lsb6p-Sepharose were constituted on ice. Reactions were initiated by the addition of [γ - ^{32}P]ATP (approx. 10 mCi/mmol) and MgCl_2 to final concentrations of 2 and 15 mM respectively, and were performed at 30°C . After 1–15 min (within the linear range of the assay), the reaction was terminated by the addition of a chloroform/methanol/HCl mixture. Lipids were extracted and separated by TLC. Spots, corresponding to PI(4)P, detected by autoradiography, were scraped, and radioactivity was measured in a liquid-scintillation counter. In some experiments with the purified enzyme, the lipid extract was counted directly after the termination of the assay. Results were essentially identical whether lipids were separated by TLC or not. Kinetic data were modelled to fit the Michaelis–Menten equation using a non-linear least-squares fit, with the Hill coefficient set to 1, using Prism v.3 software (Graphpad Software, San Diego, CA, U.S.A.).

Analysis of Lsb6p substrate specificity

To determine if Lsb6p has both PI kinase and PI(P) kinase activities, His₆-Lsb6p was assayed as described above using PI, PI(3)P, PI(4)P and PI(5)P as substrates. Lipids were separated by TLC and autoradiography was performed to identify radioactive products [PIP and phosphatidylinositol 4,5-bisphosphate (PIP₂)]. To confirm that *LSB6* encodes a PI 4-kinase, a PI kinase reaction with His₆-Lsb6p was performed as described above, and lipids were extracted, deacylated and subjected to HPLC as described previously [22]. For assaying PIPs in yeast overexpressing or lacking Lsb6p, the wild-type or Δlsb6 yeast strain containing pRS426 or pRS-426-PGK-Pik2p was subjected to radiolabelling with [^3H]myo-inositol as described previously [22], before extracting lipids and processing samples for HPLC.

RESULTS

A comparison of primary sequence from Lsb6p with a representative mammalian Type II kinase, namely the Type II α enzyme from rat [4], is shown in Figure 1. The yeast protein is 27 % larger, primarily due to large inserts within the catalytic domain, which resides in the C-terminal portion of the kinase (residues 161–607, by analogy with rat PI4KII α [23]). Mammals have two isoforms of PI4KII, namely α and β , which differ mainly at the N-terminus upstream of the predicted catalytic domain [5]. The N-terminal region of Lsb6p (residues 1–160) has no sequence similarity with the corresponding regions of either form of mammalian PI4KII (approx. residues 1–125). However, the catalytic domains have an overall similarity/identity of

Figure 1 Sequence similarity of yeast and rat PI4KII

The *S. cerevisiae* *LSB6*-encoded protein is compared with the rat PI 4-kinase Type II α . Three regions of high sequence similarity are noted by dark and light grey rectangles and by thin boxes at the C-terminus. The glycine-rich loop and key residues predicted to be involved in substrate binding and catalysis (see text) are indicated. The C-terminal amino acids are also indicated. NS, no apparent similarity.

Figure 2 *LSB6* encodes a PI kinase

High- and low-copy plasmids containing the sequence for myc-Lsb6p were expressed in wild-type yeast. Lysates derived from transformed culture were obtained and exposed in triplicate (duplicate for wild-type) to anti-myc beads. The beads were isolated and subjected to assay for PI kinase. The reaction product from purified rat enzyme, shown on the right, was used as a PIP marker.

51 %/35 %. Two segments, located within residues 165–251 and 363–514, are especially conserved between the mammalian and yeast kinases (Figure 1). Within the putative catalytic domain of Lsb6p, there is a ‘kinase core’ region (residues 161–419) that contains several conserved amino acids, which, by analogy to other lipid and protein kinases [24], are likely to be essential for substrate binding and catalysis. The N-terminal portion of this

Figure 3 Lsb6p is a PI 4-kinase

His₆-Lsb6p was purified from Sf9 cells (inset), and assayed for PI 4-kinase activity as described in the Materials and methods section. Phospholipids were isolated and deacylated to release inositol sugars, which were then subjected to HPLC. The indicated standards represent molecules before the deacylation step. The major product of Lsb6p co-migrates exactly with the PI(4)P standard. Residual ATP substrate from the reaction is visible on the right of the chromatogram.

kinase core contains the glycine-rich loop, ¹⁷⁰GSSGSYFV¹⁷⁷, which is strictly conserved among PI4KII family members from mammals, *Drosophila*, *Caenorhabditis elegans* and *Schizosaccharomyces pombe*, as well as a lysine residue (Lys¹⁹²), which, in other kinases, anchors the α and β phosphates of ATP. The C-terminal portion of the core contains an aspartic acid residue (Asp³⁸⁷), which may function as a catalytic base, and two residues involved in binding Mg²⁺, an asparagine residue (Asn³⁹²) and an aspartic acid residue (Asp⁴¹³). Deletion of only seven residues from the C-terminus of rat PI4KII α reduces the enzymic activity by almost 80% [23]. The similarity between the C-termini of the yeast and rat enzymes (PVFTWC for yeast, PFFSWW for rat) suggests a common function in this region. The yeast genome does not contain any other recognizable member of PI4KII.

To estimate the contribution of LSB6p to cellular PI production, we first analysed PIPs in cell lysates from strains lacking *LSB6* or overexpressing Lsb6p and radiolabelled with inositol. Disrupting the gene had no significant effect on PI(4)P levels (results not shown). Similarly, there was no major reproducible increase in the levels on overexpression (results not shown). We attribute our results to relatively high activities of Pik1p and Stt4p, such that Lsb6p, even when overexpressed, could not contribute significantly to the bulk pool of PI(4)P. Also, homeostatic mechanisms operate probably to prevent drastic elevations in cellular PI levels. Indeed, when we overexpressed the rat Type II kinase in mammalian cells at 10- to 20-fold higher levels than all forms of endogenous enzymes (both Types II and III), no increase in cellular PI(4)P levels was detected (B. Barylko, D. D. Binns and J. P. Albanesi, unpublished work).

To assay the activity of the kinase directly, we first tagged Lsb6p with the myc epitope at its N-terminus and expressed it in wild-type yeast cells on a single- or multi-copy plasmid. Lysates from transformed cells were then immunoprecipitated with anti-myc beads, and the pellets were subjected to a PI kinase assay measuring transfer of phosphate from [γ -³²P]ATP to PI and identifying the product on TLC plates. Activity was barely detectable from untransformed cells and was slightly increased in cells containing the single-copy plasmid (Figure 2). In contrast,

Figure 4 Lsb6p does not phosphorylate PIP species

His₆-Lsb6p, purified from Sf9 cells, was incubated with 1 mM of the indicated lipid for 5 min and then assayed for activity. The position of standards is shown on the left of the image of the TLC plate. Duplicate samples are shown.

cells expressing Lsb6p on a multi-copy plasmid contained an average of 9-fold higher activity compared with wild-type cells. The product co-migrated with that produced from the purified rat enzyme, suggesting that Lsb6p is indeed a PI 4-kinase.

To establish further that Lsb6p had PI 4-kinase activity, it was tagged with His₆, expressed in Sf9 insect cells and purified from detergent lysates (Figure 3, inset). Similar to rat PI4KII α , which is an integral membrane protein due to palmitoylation in a cysteine-rich motif [4], the yield of His₆-Lsb6p was much lower if detergents were not used. The purified, tagged enzyme was assayed in the presence of [γ -³²P]ATP. Products were subjected to deacylation and HPLC. The major lipid product co-migrated exactly with the headgroup of a PI(4)P standard (Figure 3). Therefore we concluded that *LSB6* encodes an authentic PI 4-kinase.

To determine whether Lsb6p can also phosphorylate PIP substrates, it was incubated with PI(3)P, PI(4)P and PI(5)P. No PIP₂ was produced with any of these substrates (Figure 4). A low level of PIP was detected using PI(3)P, which we attribute to contaminating PI in this commercial preparation.

Kinetic parameters for the purified Lsb6p are generally consistent with those of a Type II kinase. However, the K_m value for ATP (266 μ M, Figure 5A) was intermediate between values of mammalian Type II and III enzymes (< 100 and > 400 μ M respectively [25]).

The presentation of the other enzyme substrate, PI, on a membrane or micellar surface complicates kinetic measurements. As is done for a model of surface dilution kinetics [26], we chose to determine activities at different lipid/detergent ratios and to extrapolate V_{max} for pure lipid. Enzyme assays were performed in Triton X-100-containing buffer such that the detergent/PI ratios were 5:1, 10:1, 20:1, 40:1 and 60:1. Enzyme activity as a function of decreasing ratios is shown in Figure 5(B). As expected, the apparent V_{max} values increased in inverse proportion to the detergent/PI ratio, since the enzyme requires fewer micelle-binding-dissociation events as more PI is packed within each micelle. Plotting the apparent V_{max} values as a function of the lipid/(detergent + lipid) ratio (Figure 5C) allowed us to estimate a catalytic-centre activity of 125 min⁻¹ for a pure PI system.

Type II and III PI 4-kinases are distinguishable by differential sensitivities to inhibition by wortmannin (which inhibits the Type III but not Type II enzyme) and adenosine (which inhibits Type II and III enzymes with a K_i value of approx. 20 μ M and approx. 1.5 mM respectively) [3,25]. The yeast enzyme is not

Figure 5 Enzymic characterization of purified His₆-Lsb6p

(A) Dependence on ATP. The points were modelled to the Michaelis–Menton equation (Hill coefficient set at 1) and they indicate a K_m value of 0.266 mM for ATP. (B) Dependence on PI at detergent/PI ratios of 5:1, 10:1, 20:1, 40:1 and 60:1 as indicated. The concentration of ATP in this experiment was 2 mM. The data were modelled as in (A). (C) The V_{max} values obtained from the data in (B) are plotted against (detergent + PI)/PI values. The curve extrapolates to a catalytic-centre activity of approx. 125 min⁻¹ at pure PI. (D) Inhibitory effect of adenosine, measured at 0.3 mM ATP. Error bars represent means $\pm$ S.E. from triplicate measurements (duplicates for the ATP curve); absence of error bars indicate that they lie within the symbols.

affected by wortmannin (results not shown) but is inhibited approx. 80% by 1 mM adenosine (IC_{50} approx. 150 μM), consistent with its being a Type II enzyme (Figure 5D), although sensitivity to adenosine is weaker for the yeast enzyme than for mammalian enzymes.

To gain information on the localization of Lsb6p, we tagged the protein with the HA epitope at the N-terminus of Lsb6p and expressed it from a low-copy plasmid. Fractionation of yeasts revealed that virtually all of the immunoreactivity was particulate (as we observed in Sf9 cells) and most of it was pelletable even at low speed, suggesting that it is contained in large organelles (Figure 6). Under these lysis conditions, less than half of the mitochondria and peroxisomes pellet in a 550 g spin ([27] and results not shown). As expected, most of the cytoplasmic marker GADPH was in the supernatant in all cases. A variable amount of the kinase, typically about half, could be extracted from these membranes with either Triton X-100 or sodium carbonate (results not shown), making conclusions difficult regarding orientation in the membrane. Expressed mammalian PI4KII α cannot be extracted without detergent, whereas most of the PI4KII β can

be extracted with carbonate [28]. Thus yeast Lsb6p may utilize two modes of membrane attachment. Protein tagged at the C-terminus of Lsb6p with GFP yielded the same behaviour as N-terminal HA-tagged protein (results not shown).

We found that expression of these constructs was highly variable from experiment to experiment. Although a high expression promoter (from the yeast phosphoglycerate kinase gene) was used in our studies, protein was often difficult to detect, regardless of the epitope tag employed. Cells tended to lose protein expression quickly with passaging on plates or in liquid, although the plasmid was maintained, suggesting that the expression of the kinase is kept under close post-transcriptional control or that the protein is toxic.

Two organelles that pellet at low speeds from lysates are the plasma membrane and vacuole [29]. Indeed, the GFP-tagged construct localized to both of these locations (Figure 7). The dye FM4-64 is endocytosed from plasma membrane and is a useful marker of the vacuole (Figure 7, middle panels) [20]. These results suggest a dual localization of the kinase to plasma membrane and vacuole. Somewhat similarly, the mammalian

Figure 6 HA-Lsb6p is particulate and present in large membranes

Cells expressing HA-Lsb6p were converted into spheroplasts, osmotically lysed and subjected to centrifugation at 550, 2200 or 150 000 *g* as indicated. Equivalent supernatants (S) and pellets (P) were subjected to SDS/PAGE, blotted and exposed to antibody directed against the HA epitope or the cytoplasmic marker GAPDH. SM, equivalent fraction of starting material (lysate).

Type II enzyme has been localized very recently to early endosomes and other internal vesicles [30]. However, the FM4-64-generated vacuolar pattern was unchanged in the *LSB6* disruption strain (results not shown), ruling out an essential function for *LSB6* in general membrane endocytosis.

In an attempt to define the function for *LSB6*, we subjected the disruption strain to rich or minimal media at normal or elevated temperatures, with various carbon sources (glucose, raffinose or acetate), or in the presence of various stressors (1 M NaCl, 25 mM RuCl₃, 5 mM MnCl₂ or 6 mM ZnCl₂), but we found no

variance in growth from the wild-type strain. We found that the strain was capable of inhibiting the growth of the opposite mating type similar to wild-type, and spores not containing *LSB6* were capable of germinating normally. Although we were not able to identify a phenotype of the *LSB6* disruption strain, the localization of the tagged kinase to plasma membrane and vacuole indicates that a role for it, probably subtle, in endocytosis or exocytosis is a reasonable possibility for its function.

DISCUSSION

We have expressed Lsb6p, the yeast orthologue of the recently cloned rat PI4KII, and confirmed that it has PI 4-kinase activity. As expected for a Type II enzyme, it is sensitive to adenosine but not to wortmannin. However, the ATP dependence of Lsb6p, with $K_m = 266 \mu\text{M}$, is quite high compared with other Type II enzymes; for example, the K_m values for the rat enzyme is only $88 \mu\text{M}$ [4]. However, at normal cytosolic ATP concentrations, this difference may have negligible functional significance.

Similar to the model of surface dilution kinetics [26], the velocity of product formation increased at a fixed PI concentration with an increase in the ratio of PI to detergent, presumably because more substrate can be phosphorylated on the same mixed micelle. The theoretical $V_{\max}$ in pure PI, obtained by extrapolation, was approx. 125 min^{-1} . This is quite similar to the activity of the rat enzyme, where the catalytic-centre activity (at fixed lipid/detergent ratio) was at least 150 min^{-1} [23]. We suspect that the enzymic rate may be modified by cellular factors, perhaps binding to the large N-terminal segment, which is highly diverse in sequence among all putative Type II kinases. A screen for factors that interact with this region may be fruitful in understanding the regulation of this enzyme.

Our expressed enzyme is membrane-bound. Fractionation experiments show that HA- or GFP-tagged molecules precipitate

Figure 7 Lsb6p-GFP localizes to yeast plasma membrane and vacuolar membrane

Lsb6p-GFP expressed on a low-copy plasmid was visualized in viable cells by fluorescent microscopy. Vacuolar membrane was labelled by the red dye FM4-64. The arrows show vacuoles in three cells. The three arrowheads show cells with localization to both the vacuolar membrane and cell surface.

with large membranes. This is consistent with the GFP localization by fluorescence microscopy, showing that the protein localizes both in vacuoles and in plasma membrane [29]. It is interesting that LAS17p, the protein to which LSB6 has been found to bind in a two-hybrid assay [13], also localizes to vacuoles and has been found to participate in homotypic vacuolar fusion [31]. However, whereas *LAS17* is required for *in vitro* vacuolar fusion and normal vacuolar morphology *in vivo* [31], deletion of *LSB6* does not lead to any obvious defects in vacuolar morphology (Figure 7). If the kinase activity is indeed contributing to vacuolar biogenesis, its role may be subtle.

Nickels et al. [32] reported two PI 4-kinase activities on yeast membranes. These activities corresponded to proteins of 45 and 55 kDa when purified, and are, apparently, unrelated to Pik1p and Stt4p, the two soluble yeast Type III kinases. Both forms have a similar specific activity to Lsb6p and, like Lsb6p, are particulate enzymes. However, the amino acid analysis [32] and apparent molecular mass on SDS gels are not consistent with the predicted composition of Lsb6p. For example, the number of lysine residues determined in the 45 and 55 kDa species were 1.8 and 0 ('not detected'), whereas there are 38 lysine residues in Lsb6p, which are distributed throughout the primary sequence. These results indicate that yeast may have other as yet uncloned PI 4-kinases.

Our localization is somewhat similar to that observed with the mammalian enzyme, which is found in the plasma membrane, small vesicles adjacent to plasma membrane, lysosomes and Golgi in cultured cells [28,30]. However, we have failed so far to detect any defects in membrane transport pathways in our Lsb6p disruption strain. Therefore the function of the protein is either redundant or specific to substrates, growth conditions or developmental pathways not yet assayed.

Results of Audhya et al. [11] indicate that the two PI 4-kinases identified previously, namely Pik1p and Stt4p, provide at least 95% of total cellular PI(4)P. Consistent with this, we observed no difference in PI(4)P pools in the *LSB6* disruption strain. However, since pools generated by Pik1p and Stt4p seem to be in segregated compartments, it is possible that PI(4)P generated by Lsb6p is isolated in a third cellular compartment. Identification of such a compartment may be difficult, but it may be possible to fractionate organelles from cells that are temperature-sensitive in both *PIK1* and *STT4* [11], with or without the *LSB6* allele, to look for such a pool. Such an approach may yield further insight into the function of this novel yeast kinase.

Note added in proof (received 17 February 2003)

While our manuscript was in review, Han et al. [33] reported that *LSB6* encoded a PI 4-kinase. Most of this work is in agreement with our studies. The activity of the expressed enzyme purified from *Escherichia coli* was found to be approx. 30000-fold lower than ours, which is probably due to the lack of a post-translational modification in bacteria; the mammalian enzyme and also probably the yeast enzyme are subjected to palmitoylation, which is essential for high activity [4].

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