A Phosphatidylinositol/Phosphatidylcholine Transfer Protein Is Required for Differentiation of the Dimorphic Yeast *Yarrowia lipolytica* from the Yeast to the Mycelial Form

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Abstract. The SEC14Sc gene encodes the phosphatidylinositol/phosphatidylcholine transfer protein (PI/PC-TP) of *Saccharomyces cerevisiae*. The SEC14Sc gene product (SEC14pSc) is associated with the Golgi complex as a peripheral membrane protein and plays an essential role in stimulating Golgi secretory function. We report the characterization of SEC14vL, the structural gene for the PI/PC-TP of the dimorphic yeast *Yarrowia lipolytica*. SEC14vL encodes a primary translation product (SEC14pVL) that is predicted to be a 497-residue polypeptide of which the amino-terminal 300 residues are highly homologous to the entire SEC14pSc, and the carboxy-terminal 197 residues define a dispensible domain that is not homologous to any known protein. In a manner analogous to the case for SEC14pSc, SEC14pVL localizes to punctate cytoplasmic structures in *Y. lipolytica* that likely represent Golgi bodies. However, SEC14pVL is neither required for the viability of *Y. lipolytica* nor is it required for secretory pathway function in this organism. This nonessentiality of SEC14pVL for growth and secretion is probably not the consequence of a second PI/PC-TP activity in *Y. lipolytica* as cell-free lysates prepared from Δsec14vl strains are devoid of measurable PI/PC-TP activity in vitro. Phenotypic analyses demonstrate that SEC14pVL dysfunction results in the inability of *Y lipolytica* to undergo the characteristic dimorphic transition from the yeast to the mycelial form that typifies this species. Rather, Δsec14vl mutants form aberrant pseudomycelial structures as cells enter stationary growth phase. The collective data indicate a role for SEC14pVL in promoting the differentiation of *Y. lipolytica* cells from yeast to mycelia, and demonstrate that PI/PC-TP function is utilized in diverse ways by different organisms.

All eukaryotic cells have the ability to execute both protein and lipid sorting events. While much has recently been learned about the mechanisms by which proteins traffic between intracellular compartments, or by which proteins are retained in specific organelles, considerably less is known about the intracellular trafficking of lipids. Yet, it is obvious that lipid traffic must also encompass a set of essential cellular activities. For example, intracellular organelles exhibit unique lipid compositions (van Meer, 1989; Pagano, 1990). Moreover, whereas the ER represents the major compartment of lipid synthesis in the eukaryotic cell, other intracellular compartments that experience a significant influx of ER-deprived lipids (e.g., Golgi complex and mitochondria) nevertheless manage to maintain characteristic lipid compositions in their respective membranes. As the specific lipid composition of an organelle is likely to play an important role in determining organelle function, the demonstration of lipid heterogeneity between distinct organelle membranes indicates a role for lipid sorting in the establishment and maintenance of compartmental identity within the cell. Lipid trafficking is also likely to be essential for the maintenance of organelar integrity, especially in the case of the ER which provides the bulk lipid that sustains vesicle-mediated protein traffic through the secretory pathway. Wieland et al. (1987) have argued that massive retrieval of bulk lipid from the Golgi back to the ER is required to spare the latter from rapidly consuming itself in the process of donating lipid to later stages of the secretory pathway.

Four general mechanisms for intracellular lipid traffic have been entertained (reviewed in Bishop and Bell, 1988;
Phospholipid transfer proteins; PS, phosphatidylserine. BHT, butylated hydroxytoluene; PC-TP, phosphatidylcholine transfer protein; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL-TP, phospholipid transfer proteins; PS, phosphatidylserine.

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Materials and Methods

Strains, Media, and Genetic Methods

A description of the plasmids and genotypes of the yeast strains used in this study is given in Table I. The Y. lipolytica strains used in this study were derived from three distinct haploid strains: EI22, JM12, and W29 (Table I). Standard complex and minimal media included YPD and YNB medium, respectively (Sherman et al., 1986). In experiments where secretion of alkaline protease or acid phosphatase was determined, cells were grown on YPDm and low-Pi medium, respectively (Nicaud et al., 1989; Lopez and Dominguez, 1984). Escherichia coli K-12 strains TG1 and HB101 were routinely employed for propagation of plasmids, and were cultured on standard LB and 2XYT media (Sambrook et al., 1989).

Yeast genetic techniques employed published procedures. Integrative transformation of Y. lipolytica with linearized plasmids was accomplished by the lithium acetate procedure of Xuan et al. (1990), while routine introduction of ARS-CEN plasmids into Y. lipolytica was via electroporation (Fournier et al., 1993). The authenticity of integration events, or other allelic replacement events, was routinely confirmed by Southern hybridization analysis.

Recombinant DNA Methodologies

Recombinant DNA techniques were performed essentially as described by Sambrook et al. (1989). A Y. lipolytica cDNA library consisting of 18,000 clones, with an average insert size of 1.6 kb, was generated from strain W29 in the S. cerevisiae expression vector pFL61. This vector is a 2 #m circle plasmid that carries the URA3 gene for selection purposes and provides the yeast PGK promoter to drive the strong constitutive expression of cloned DNA (Minet et al., 1992). RNA prepared from strain W29 growing exponentially on YPD, was converted to cDNAs which were subsequently linked to BstXI adaptors prior to their insertion into BstXI-digested pFL61. Recombinant DNA techniques were performed essentially as described by Sambrook et al. (1989). A Y. lipolytica cDNA library consisting of 18,000 clones, with an average insert size of 1.6 kb, was generated from strain W29 in the S. cerevisiae expression vector pFL61. This vector is a 2 #m circle plasmid that carries the URA3 gene for selection purposes and provides the yeast PGK promoter to drive the strong constitutive expression of cloned DNA (Minet et al., 1992). RNA prepared from strain W29 growing exponentially on YPD, was converted to cDNAs which were subsequently linked to BstXI adaptors prior to their insertion into BstXI-digested pFL61.

The primers used to amplify a segment of SEC14p were designed taking into account the primary sequences inferred from nucleotide sequence analysis of the respective genes; and (b) hybridization with linearized plasmids was accomplished by the lithium acetate procedure of Xuan et al. (1990), while routine introduction of ARS-CEN plasmids into Y. lipolytica was via electroporation (Fournier et al., 1993). The authenticity of integration events, or other allelic replacement events, was routinely confirmed by Southern hybridization analysis.

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### Table 1. Plasmids and Strains Used

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Source or reference</th>
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<tr>
<td>pCTY11</td>
<td><em>S. cerevisiae</em> YEp vector carrying LEU2, ADE3, and SEC14</td>
<td>V. Bankaitis, 1992</td>
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<td>pFL61</td>
<td><em>S. cerevisiae</em> expression vector based on PGK promoter and terminator, carrying URA3</td>
<td>Minet et al., 1992</td>
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<td>pINA62</td>
<td>5.6-kb Sall fragment carrying LEU2(^{NT}) in pBR322</td>
<td>Gaillardin and Ribet, 1987</td>
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<td>pINA237</td>
<td>pBR322 carrying LEU2(^{NT}) and ARS-CEN18</td>
<td>Fournier et al., 1993</td>
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<td>pINA300'</td>
<td>1.5-kb Sall fragment carrying URA3(^{NT}) in pBR322</td>
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</tr>
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<td>pINA476</td>
<td>XPR2 terminator and LEU2(^{NT}) gene in pBR322</td>
<td>Tharaud et al., 1992</td>
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<td>pINA540</td>
<td>7.2-kb Sau3A fragment carrying SEC14(^{NT}) in pINA62</td>
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<td>pINA651</td>
<td>3.6-kb HindIII-Sau3A fragment carrying SEC14(^{NT}) in pINA62</td>
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<td>pINA653</td>
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<td>pINA656</td>
<td>plNA300' with filled-in EcoRI site</td>
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<td>pINA929</td>
<td>2.7-kb Stul-Ball deletion of SEC14(^{NT}) in pBR322</td>
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<td>pINA930</td>
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<td>pRE510</td>
<td><em>S. cerevisiae</em> SEC14 cDNA in pTZ18</td>
<td>D. Malehorn and Bankaitis</td>
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<td>Strain</td>
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<td>CTY1-1A</td>
<td>MATa, ura3-52, Δhis3-200, lys2-801, sec14-1</td>
<td>McGee and Bankaitis</td>
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<td>CTY558</td>
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<td>Y. lipolytica</td>
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<td>W29</td>
<td>MATa</td>
<td>Wild type (our collection)</td>
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<td>Fabre et al., 1992</td>
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<td>PO1a</td>
<td>MATa, Δleu2-270, Δura3-302</td>
<td>W29 derivative, Tharaud et al., 1992</td>
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<td>JM12</td>
<td>MatB, leu2-35, lys5-12, ura3-18</td>
<td>Nicaud et al., 1989</td>
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<td>MCL8</td>
<td>MatA/MatB, +/Δleu2-270, +/lys11-23, +/his-1, Δura3-302, Δura3-302, SEC14/sec14::URA3</td>
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<td>E122 + pINA653</td>
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<td>MCL9 + HindIII-Sall fragment of pINA652</td>
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<td>E122 derivative, see Material and Methods</td>
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<td>MatB, lys5-12, ura3-18</td>
<td>JM12 + NotI-digested pINA62</td>
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<td>MCL29</td>
<td>MatB, leu2-35, lys5-12, ura3-18</td>
<td>JM12 + HindIII-Sall fragment of pINA652</td>
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<td>MCL29 + NotI-digested pINA62</td>
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<td>MCL41</td>
<td>MatA, Δleu2-270, Δura3-302, sec14Δ2::LEU2</td>
<td>PO1a + SphI fragment of pINA930</td>
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of *Y. lipolytica* DNA, the PCR reactions contained 150 ng of DNA, and 25 pmol of each primer in a final reaction volume of 50 μl. 30 amplification cycles (94°C, 20 s; 45°C, 1 min; 72°C, 1 min) were conducted using a GeneAmp PCR reagent kit (Perkin-Elmer Corp., Norwalk, CT). The 219-bp PCR product was purified on an agarose gel, rendered blunt-ended by treatment with T4 DNA polymerase, and subcloned into the unique EcoRV site of Bluescript\(^{TM}\) KS or SK vectors (Stratagene Corp., La Jolla, CA). This cloned PCR product was used as a probe to screen a genomic library of *Y. lipolytica* DNA (Xuan et al., 1990). The DNA sequence of SEC14\(^{NT}\) was obtained by the method of Sanger et al. (1977), and the nucleotide and inferred primary sequence data were analyzed using Version 7 of the UWGCG package (Devereux et al., 1984).

Three different mutant sec14\(^{Δ2}\) alleles were constructed. First, pINA652 (see Fig. 2 b) carries a simple sec14\(^{Δ2}\)::URA3 disruption allele. This plasmid was constructed by inserting the URA3\(^{NT}\) gene, as a 1.5-kb Sall restriction fragment (from pINA300' ; see Table I), into the unique XhoI site present in pINA651, thus interrupting the SEC14\(^{NT}\) coding sequence 162 codons downstream of the initiator codon. Second, the sec14\(^{Δ2}\)::Δ allele represents a 0.4-kb deletion bounded by the NruI and XhoI sites of SEC14\(^{NT}\), and this allele is carried on pINA657 (see Fig. 2 c). This plasmid was constructed by ligating the ClaI-NruI (blunted) and the XhoI (blunted)-cleaved plasmid to yield pINA657. Subsequent insertion of a 3.1-kb PstI fragment carrying LEU2\(^{NT}\) into the ClaI-SphI fragments of pINA651 into plNA300' yielded the sec14\(^{Δ2}\)::Δ plasmid pINA929 (see Fig. 2 d). Replacement of the genomic SEC14\(^{NT}\) gene with the sec14\(^{Δ2}\)::Δ allele was via a two step gene substitution protocol (Boecke et al., 1987). Plasmids were targeted to the
genomic $SEC14^{pl}$ locus, and Ura* transformants of Y. lipolytica were selected. The transformants were challenged with 5-fluoroorotic acid (1.25 mg/ml) on YNB medium supplemented with uracil (10 μg/ml) to select for strains cured of vector sequences by homologous recombination between $SEC14^{pl}$ sequences flanking the integrated vector. The recombinants in which the desired gene replacement event had occurred were identified by Southern analysis (Table I).

**Immunoochemical Techniques**

The kinetics of transport of the alkaline extracellular protease (AEP) was monitored in the appropriate strains by pulse–chase experiments followed by immunoprecipitation of AEP from cell-free extracts prepared from cells harvested at various times post-chase as described by Fabre et al. (1992). A polyclonal rabbit anti-SEC14p$^{pl}$ serum raised against a TrpE-SEC14p$^{pl}$ fusion protein (Bankaitis et al., 1989) was used to visualize SEC14p$^{pl}$ by immunoblotting. Yeast cells were grown to stationary phase in YNB at 28°C, and immunoblotting was performed as described by Fabre et al. (1991). The anti-SEC14p$^{pl}$ serum was used at a 1:500 dilution and the immunoblots were developed with an alkaline phosphatase-conjugated secondary antibody obtained from Promega Biotech (Madison, WI) (1:10,000 dilution).

Immunofluorescence experiments were performed as previously described (Pringle et al., 1989; Cleves et al., 1991b). Y. lipolytica cells were grown in minimal medium to mid-logarithmic phase, and the cells were fixed in situ by incubation with formaldehyde (3.7% final concentration) for 1 h at room temperature, and incubated overnight at 4°C to allow further fixation. The fixed cells were converted to spheroplasts and attached to coverslips by a 5 min centrifugation at 1,000 g in a Cytospin 2 centrifuge (Shandon Inc., PA). Cells were subsequently immersed in ice-cold methanol (5 min), rinsed in ice-cold acetone (30 s), flooded with blocking buffer (0.01% Tween 20, 1% BSA in phosphate buffered saline), and incubated with mouse anti-KEX2p and rabbit anti-rat PI-TP antibodies in blocking buffer at concentrations of 44 and 49 μg/ml, respectively. Spheroplasts were exhaustively washed in blocking buffer and incubated with sheep anti-mouse antibodies (15 μg/ml) for 2 h. This step permitted further decoration of bound mouse antibodies and amplification of the mouse antibody-dependent (i.e., XPR6p) immunofluorescence signal. After another round of extensive washing with blocking buffer, cells were incubated in the presence of Texas red-conjugated donkey anti-sheep and ~-conjugated donkey anti-rabbit antibodies (Jackson ImmunoResearch, West Grove, PA) for 2 h at a concentration of 30 μg/ml each. After a final wash in blocking buffer, the staining profiles were visualized with a Nikon Optiphot epifluorescence microscope equipped with differential interference contrast optics and a Dage series 68 ST video camera (Dage-MTI Inc., Wabash, IN) coupled to an Image-1 analysis system (Universal Imaging, Westchester, PA). Image processing was performed as described by Wang et al. (1993). The images were printed with a Sony UP-5000 color video printer (Sony, Muntvale, NJ).

**Phospholipid Transfer Assays**

Cells were harvested from exponential or stationary phase Y. lipolytica cultures that had been grown in either YPD or YNB medium. Cell pellets were washed twice with 0.4 M sucrose, 6 mM EDTA, 1 mM cysteine, 9 mM 2-mercaptoethanol, 0.1 M Tris-HCl, pH 7.5. The cells were disrupted by mechanical agitation in the presence of glass beads using a Braun-Melsungen™ cell homogenizer. Cell lysates were clarified by centrifugation at 12,000 g for 15 min and the resulting supernatant was further centrifuged at 100,000 g for 1 h. The supernatant was collected and brought to 75% saturation by the slow addition of powdered ammonium sulfate with gentle stirring. After a 3-h incubation at 4°C, the resulting precipitate was collected by centrifugation at 8,000 g for 30 min, and resuspended in a minimal volume of 10 mM sodium phosphate, pH 7.2, 10% glycerol, 8 mM 2-mercaptoethanol, and 1 mM NaCl. The resulting suspension was dialyzed against 40-20 vol of the same buffer. Finally, the dialyzed was adjusted to pH 5.1, centrifuged at 8,000 g for 30 min to remove insoluble material, and readjusted to pH 7.2. The PI- and PC-transfer activities of this cytosolic fraction were measured as reported (Kader et al., 1987).

**Quantitative Phospholipid Analyses**

Pulse radiolabeling of yeast strains was performed on mid-logarithmic cultures grown in the synthetic complete medium of Klig et al. (1985) essentially as described by McGee et al. (1994). The pulse involved incubation with [32P]orthophosphate ([32P]; 10 μCi/ml) for 30 min at 25°C with shaking. For steady-state [32P]-labeling experiments, yeast were grown in synthetic complete medium overnight, subcultured, and then presented with [32P]; (10 μCi/ml) for a period of five to six cell generations at 25°C with shaking to permit steady-state labeling of cellular phospholipids (Atkinson et al., 1980; Klig et al., 1985). For both types of radiolabeling experiments, phospholipids were extracted by the method of Atkinson (1984). Yeast cells were pelleted by a low speed spin (500 g), washed in ice cold TCA (5%) for 20 min with subsequent repelleting, and the pellet resuspended in 1 ml polar extraction solvent (Steiner and Lester, 1972) with heating at 85°C for 20 min. Phospholipids were recovered from the cell suspension by a wash in CHCl3/CH3OH/butylated hydroxytoluene (BHT) (2:1:0.0005%), dried under N2 gas, and resuspended in CHCl3/CH3OH/BHT. Radiolabeled phospholipids were resolved by two-dimensional chromatography using Whatman S8G1 paper (Steiner and Lester, 1972). First dimension solvent was CHCl3/CH3OH/NH4OH/H2O (22: 9: 1: 0.26) and second dimension solvent was CHCl3/CH3OH/CH3COOH/H2O (8: 1: 1.25: 0.25). Labeled phospholipids were detected by autoradiography, and identified by comparison to commercial standards. Individual phospholipid species were cut from the chromatography paper, and were quantitated by scintillation counting.

**Enzyme Assays**

Periplasmatic acid phosphatase was measured as described by Lopez and Domínguez (1988). Cells were washed with deionized water and incubated at 30°C in 450 μl of 0.1 M maleic acid–sodium maleate buffer, pH 6.2, containing 6.7 mM p-nitrophenyl-phosphate. The reaction was terminated by adding 750 μl of 0.1 M NaOH, and the amount of p-nitrophenol released was estimated at 410 nm.

**Results**

**Isolation of the SEC14p Gene**

We used a PCR strategy to recover genomic $SEC14^{pl}$ clones and a complementation strategy to recover cDNA clones of $SEC14^{pl}$ (see Materials and Methods). A 219-bp fragment of Y. lipolytica DNA was amplified that had the potential to encode an open reading frame sharing 74% identity with the expected portion of the SEC14p$^{pl}$ primary sequence. This PCR product was then used as a probe for the in situ screening of a plasmid genomic library of Y. lipolytica DNA propagated in E. coli. Three identical plasmids, designated pINA540, were isolated from a total of 15,000 clones screened, and a 3.9-kb HindIII-Sau3A fragment containing the entire SEC14p gene was subcloned into pBR322 to generate pINA651 (Table I).

The Y. lipolytica cDNA expression library was transformed into the ura3-52, sec4-1 + S. cerevisiae strain CTY1-1A and Ura* transformants were selected at 25°C, a permissive temperature for sec4-1 + yeast strains. A total of approximately 20,000 Ura* transformants were screened for growth at a temperature restrictive for sec4-1 + by replica plating onto uracil deficient minimal medium and incubation at 37°C. Two colonies capable of such growth were obtained, and two criteria were employed to demonstrate that the unselected Ts* phenotype was due to a plasmid-linked trait. First, isolation and characterization of spontaneous segregants that had lost plasmid under nonselective conditions revealed that plasmid-cured derivatives failed to grow at 37°C. Second, plasmids were recovered from the two Ts* Ura* transformants by transformation into E. coli. Subsequent transformation of CTY1-1A with each of the two isolated plasmid clones revealed a complete coincidence of inheritance of both Ura* and Ts* in the transformants. Restriction analysis of both sec4-1 + complementing cDNA indicated that the two plasmids carried identical cDNA in-
erts of approximately 1.6 kb; a result confirmed by nucleotide sequence analysis (see below). Henceforth, these cDNAs will be considered under a single plasmid designation, pINA926 (Table 1). Moreover, the restriction maps of the cDNA clones corresponded closely to that deduced for the candidate genomic SEC14pL clone identified by in situ hybridization.

These data suggested that the SEC14pL genomic and cDNA clones identified a Y. lipolytica homolog of SEC14pC. This was further confirmed by plasmid shuffle/colony sectoring experiments designed to test the ability of the cloned cDNAs to complement, or suppress, the lethality associated with the candidate genomic SEC14pL clone identified by in situ hybridization.

The sequences of both the SEC14pL cDNA insert of pINA926 and the genomic clone represented by pINA651 were determined. The nucleotide sequence of the 3.1-kb PstI-Sau3A restriction fragment derived from the pINA651 insert is presented in Fig. 1. A single open reading frame with the potential to encode a 491-residue polypeptide extending from nucleotides 1,430 to 2,902 was detected. However, no obvious initiation codon was identified because of the presence of an ochre termination codon at position 1,427 of the nucleotide sequence. This finding raised the possibility of at least one intron within the genomic SEC14pL clone. To further clarify the physical organization of SEC14pL we compared the genomic and cDNA sequences. The SEC14pL cDNA sequence revealed an insert of 1,602 bp that was terminated by a run of 32 consecutive A residues and exhibited a 1,491-nucleotide open reading frame. These data were consistent with Northern analyses that indicated SEC14pL to encode an mRNA of approximately 1.6 kb (not shown). The initiator codon identified on the cDNA sequence corresponded to an ATG at position 419 of the genomic sequence. The cDNA sequence also indicated that transcription initiated at least 39-nucleotides upstream of the initiator codon, and terminated 12-nucleotides upstream from a (TAG...TAGT...TTT) transcription termination consensus sequence identified by Zaret and Sherman (1982) in S. cerevisiae that is also a common feature of Y. lipolytica genes (Fig. 1; Strick et al., 1992). Thus, the composite nucleotide sequence data indicated that the primary SEC14pL transcript was at least 2,590 nucleotides in length and contained two introns near the 5′ end of the message (Fig. 1). The first intron spanned 465 nucleotides and was positioned between SEC14pL codons 6 and 7. The second intron spanned 526 nucleotides and initiated eight nucleotides downstream from the 3′ end of the first intron, within codon 9 of SEC14pL. The 5′-splice sites of both introns corresponded to a GTGAGTpu motif which diverges from the consensus GTAGTG 5′-splice motif of S. cerevisiae at the third and fourth positions. This diverged 5′-splice site sequence may represent a general feature of Y. lipolytica introns as the Yarrowia pyruvate kinase structural gene also contains an intron with a GTGAGTPu 5′-splice motif (Strick et al., 1992). Another feature of the first SEC14pL intron was the absence of a consensus TACTAAC box, a canonical motif that defines the site of lariat formation (Teem et al., 1984). Instead, an abbreviated TAAC box is observed (Fig. 1). We also noted that the 3′-splice acceptor CAG sequences for both SEC14pL and pyruvate kinase introns were all situated one nucleotide downstream from their corresponding TACTAAC boxes, a surprisingly close arrangement compared to S. cerevisiae (Patterson and Guthrie, 1991).

Our interpretation of the SEC14pL nucleotide sequence predicted a gene product of 497 residues (58 kD), a prediction confirmed by identification of the SEC14pL in immunoblots of Y. lipolytica cell-free extracts (see below). Thus, SEC14pL is predicted to be considerably larger than the SEC14ps of S. cerevisiae (35 kD), K. lactis (34 kD), and S. pombe (33 kD); all of which are of approximately 300 residues in length (Bankaitis et al., 1989; Salama et al., 1990). Alignment of the SEC14pL primary sequence with those of SEC14pC and SEC14pK revealed that the first 300 residues of SEC14pL shared 65 and 65.8% identities, respectively, with the full-length primary sequences of these SEC14p species. The carboxy-terminal 197 SEC14pL residues have no counterpart in SEC14pC and SEC14pK primary sequences and share no significant similarity with protein sequences currently entered in protein data bases. One notable feature of the carboxy-terminal SEC14pL domain is a proline-rich region that is followed by a leucine-rich region in which 23 leucine residues are found between residues 338 and 372 (Fig. 1).

**SEC14pL Function Is Not Essential for the Viability of Y. lipolytica**

The SEC14pL gene is essential for cell viability in S.
The nucleotide sequence of the genomic PstI-Sau3a fragment is given, as is the inferred protein sequence (in one letter code). Consensus sequences for intron splicing and transcription termination are underlined, as are potential transcription initiation elements. Vertical arrows indicate the 5'- and 3'-boundaries of the cDNA clones. The positions of PCR primers b and c used for amplification of SEC14 are indicated by horizontal arrows. Relevant restriction sites are indicated above the nucleotide sequence, and the start of the COOH-terminal SEC14p YL tail that is absent from the SEC14ps of S. cerevisiae, K. lactis, and S. pombe origin is indicated by asterisks. The SEC14 sequence data are available from EMBL/GenBank/DDBJ under accession number L20972.

To determine if SEC14 is required for spore germination, one of the SEC14 alleles of diploid strain MCL8 (Table I) was replaced by the sec14::URA3 disruption allele and the resulting heterozygote was subjected to random spore analysis (Barth and Weber, 1985). Approximately 50% of the meiotic progeny analyzed (84/200) inherited sec14::URA3, indicating that the sec14::URA3 allele did not affect Y. lipolytica cell viability. Similar experiments with sec14::Δ1 yielded the same results. Finally, we attempted direct substitution of sec14 by sec14::LEU2 allele that represents a deletion of the amino-terminal 453 residues of SEC14p YL. A variety of Y. lipolytica strains (JM12, E122, and POla) were transformed by the 4.25-kb SphI fragment from plNA930 (Fig. 2). Leu+ transformants were recovered at the usual frequencies and were confirmed by Southern analysis to have experienced the expected gene replacement (not shown). Thus, SEC14 is not essential for vegetative growth of Y. lipolytica.

The physical maps of wild-type and mutant SEC14 alleles. (a) Wild-type SEC14 cloned as a 3.9-kb HindIII-Sau3a fragment in pINA651, (b) sec14::URA3 disruption allele in pINA652, (c) sec14Δ1 allele in pINA657, and (d) sec14Δ2::LEU2 allele in pINA930. Large black boxes correspond to SEC14 YL exon domains while small open boxes define introns. Nontranscribed flanking sequences are indicated by thin lines, whereas hatched boxes represent URA3 and LEU2 in b, and d, respectively.
secl4Δ::URA3Δ as judged by their Ura+ phenotypes. Similar frequencies of inheritance were recorded for the control markers LYS5Δ (114/200) and HIS3Δ (105/200) which were also segregating in this cross. All Ura+ spores tested carried secl4Δ::URA3Δ as determined by Southern analysis, and grew at wild-type rates on both minimal and YPD media. We did note, however, that germination of the secl4Δ::URA3Δ segregants was typically delayed for approximately one day, relative to SEC14Δ progeny, regardless of whether germination occurred on minimal or YPD media (not shown). Nevertheless, these data clearly demonstrate that SEC14Δ is not an essential gene in Y. lipolytica.

SEC14pΔ Represents the Major PI/PC-TP of Y. lipolytica

We considered several possibilities for why SEC14pΔ is nonessential for Y. lipolytica viability. Included that SEC14Δ is a duplicated gene, and that SEC14pΔ is not the major PI/PC-TP of Y. lipolytica. To address the former issue, we used both nucleic acid hybridization and protein immunoblotting strategies. To search for SEC14Δ homologs at the nucleotide sequence level, we generated a radiolabeled probe by PCR using oligonucleotides b and c as synthetic primers and SEC14ΔR carried on pRES10 as template (Materials and Methods; Table I), and performed hybridizations to the appropriately digested and immobilized genomic DNAs. As demonstrated on Fig. 3a, the probe hybridized to the diagnostic 3.7-kb PstI fragment of SEC14Δ in wild-type Y. lipolytica DNA, and to the expected 3.3-kb PstI fragment of secl4ΔAl DNA. These experiments were repeated under various stringencies of hybridization with the full-length genomic SEC14Δ as probe and no other hybridizing species were detected (not shown). Although we cannot formally exclude the possibility that we failed to detect some distantly related genetic homolog of SEC14Δ, these data identify SEC14Δ as a unique gene that represents Y. lipolytica closest homolog to SEC14Δ.

Since the primary sequence of the first 300 SEC14pΔ residues share a 65% identity to that of the entire SEC14Δ (see above), we used immunoblotting to visualize Y. lipolytica polypeptides that are recognized by a polyclonal rabbit anti-SEC14Δ serum (Bankaitis et al., 1989). These antibodies identified a 58-kD SEC14Δ-immunoreactive polypeptide in lysates prepared from a wild-type Y. lipolytica strain (Fig. 3b) in agreement with predictions derived from SEC14Δ primary sequence. This 58-kD polypeptide species was not detected in lysates prepared from haploid Y. lipolytica strains harboring either the secl4Δ::URA3Δ or secl4ΔAl alleles (Fig. 3b). To determine if SEC14pΔ is the major, if not only, Y. lipolytica PI/PC-TP, we measured the PI/PC-TP activities of cytosolic fractions prepared from wild-type and secl4ΔAl Y. lipolytica strains (see Materials and Methods and Fig. 4). Wild-type Y. lipolytica cytosol exhibited a robust, protein-dependent transfer of PI and PC in the in vitro transfer assay. Under standard assay conditions, up to 12.5% of the total radiolabeled PC and 15% of the total radiolabeled PI present in donor membranes was transferred to acceptor membranes. In marked contrast, however, cytosol prepared from a secl4ΔAl mutant exhibited no significant PI- or PC-transfer activity (Fig. 4). On the basis of our experience with the PI/PC-transfer assays, we estimate that other PI-TPs capable of transferring either PI or PC would contribute less than 15% of the total cellular PI/PC-TP activity in a wild-type Y. lipolytica cell (not shown). These results were obtained regardless of whether the Y. lipolytica strains were grown in minimal or YPD medium, or whether stationary phase or logarithmic phase cultures were analyzed (not shown). The collective data indicate that the homology between SEC14Δ and SEC14pΔ extended to a conservation of PI/PC-TP activity, and identified SEC14pΔ as certainly the major PI/PC-TP of Y. lipolytica.

Y. lipolytica Is Proficient in PC Biosynthesis via the CDP-Choline Pathway

The nonessentiality of SEC14pΔ function for the viability of Y. lipolytica is in stark contrast to the essential requirement of SEC14Δ for the viability of S. cerevisiae. How-
requirement of ever, one mechanism for alleviating the essential SEC14p sc

Figure 4. Phospholipid transfer activity in lysates from wild-type and secl4nΔ strains of Y. lipolytica. PC (a) and PI (b) transfer activity was measured in cytosol prepared from the wild-type strain E122 (WT, ●), and from the secl4nΔ strain MCL12 (○). Cytosol was incubated with liposomes containing either [3H]-PC or [3H]-PC (transferable lipids), and [14C]cholesteroyl oleate (non-transferable lipid), with purified maize mitochondria (2 mg protein) for 30 min at 30°C. Mitochondria were subsequently resuspended from the liposomes by pelleting at 12,000 g for 10 min, the pellet resuspended in 2% Triton X-100, and the radioactivity measured by liquid scintillation counting. Transfer activity was expressed as percentage of radioactivity transferred from liposomes to mitochondria.

type and secl4nΔ.:LEU2n strains exhibited similar rates of synthesis for each of these four PL species. The steady state bulk membrane compositions of these strains grown in 1C- medium were similarly indistinguishable (Fig. 5 A), and consisted of 12% PI, 8% PS, 38% PC, and 30% PE. Whereas growth of the wild-type and secl4nΔ.:LEU2n strains of Y. lipolytica cultured either in medium replete with inositol and choline (1C+) or medium without inositol and choline (1C-), and radiolabeled with [32P]orthophosphate in either a 30-min pulse, or to steady-state, as indicated. Bulk cellular phospholipids were subsequently extracted, resolved, and individually quantitated as described in Materials and Methods. Quantitation of the major phospholipid species phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylserine (PS) is presented as mole percentage of total phospholipid. Minor phospholipid species such as phosphatidic acid, cardiolipin, and methylated forms of PE collectively constituted less than 1% of total phospholipid. Values for the wild-type strain (POb) are represented by the solid bars, whereas values for the secl4nΔ.:LEU2n strain (MCL41) are represented by the striped bars. These phospholipid profiles represent the averages of three independent determinations in which the deviation for each phospholipid species was less than 2% of total phospholipid.
from the Yeast to the Mycelial Form

levels of PE, as a mole percentage of total PL, than do bulk
composition in

clusion was directly confirmed by demonstrating the incorpora-
tion of [3H]choline into Y. lipolytica bulk membrane
PC, and the presence of CDP-choline pathway intermediates
(i.e., phosphocholine and CDP-choline) in Y. lipolytica
cells (not shown). These collective data demonstrate that
SEC14p sc is not involved in controlling bulk membrane
composition in Y. lipolytica, and that the nonessentiality of
SEC14p sc is not the result of a natural CDP-choline path-
way deficiency in this yeast. We also note that bulk Y. lipo-
lytica membranes contained significantly higher steady state
levels of PE, as a mole percentage of total PL, than do bulk
membranes of S. cerevisiae; an observation that has also been made for Candida albicans, another dimorphic yeast (Klig et al., 1990).

SEC14p sc Is Required for Y. lipolytica Differentiation from the Yeast to the Mycelial Form

Wild-type strains of Y. lipolytica exhibit a clear dimorphic
phenotype. On yeast nitrogen base minimal media, Y.
lipolytica grows in the yeast mode and forms elongated cells
only upon entering stationary phase. On YPD medium,
however, these cells grow as a mixture of yeast cells and
filamentous hyphae that results in a rough colony morphol-
ogy on plates (Rodriguez and Dominguez, 1984). Thus, mu-
tants unable to form hyphae are readily detected on solid
YPD medium as these form smooth colonies (Fournier et al.,
1991). We noted that all strains in which the SEC14p
allele had been replaced by secl4 sc::URA3 sc, secl4 sc::Δ1, or
secl4 sc::Δ2::LEU2 sc formed uniformly smooth colonies on
solid YPD medium while isogenic wild-type strains formed
typically rough colonies (Fig. 6 a). We also compared the
cell morphology of isogenic wild-type (MCL28) and
secl4 sc::URA3 sc (MCL30) strains pregrown in the yeast
form on liquid YNB medium and transferred to liquid YPD
medium to induce the morphological transition. As shown
in Fig. 6 b, a rapid invasion of the wild type culture by
hyphae was observed. In contrast, the secl4 sc::URA3 sc
strain was entirely deficient in the formation of elongated
cells under these growth conditions, even after prolonged
incubation. Authentic mycelium was never observed, even
when the secl4 sc::URA3 sc strain was grown on media favor-
ing hyphae formation (not shown). This strain did, however,
aquire a pseudomycelial morphology upon entry into sta-
nery phase with a concomitant formation of cell ag-
gregates (Fig. 6 b).

To confirm further that the morphological defect observed
for secl4 sc strains was the result of SEC14p sc dysfunction,
we tested whether reintroduction of SEC14p into
such strains could reverse the morphological defect. An autonom-
ously replicating LEU2 sc, SEC14 sc plasmid (pINA653; see Table 1) was transformed into a secl4 sc::Δ1 strain
(MCL12) by selection for Leu+. All such transformants regained the ability to form hyphae as evidenced by their
rough colony morphology. Moreover, subsequent curing of
pINA653 resulted in reacquisition of the mutant smooth
colony phenotype. These results indicated that the defect in
mycelium formation was a consequence of SEC14p sc dys-
function.

Subcellular Localization of SEC14p sc

SEC14p sc localizes as a peripheral membrane protein of the
S. cerevisiae Golgi complex (Cleves et al., 1991b). This as-
ignment of SEC14p sc localization is operationally defined by:
(a) the cofractionation of the membrane-associated
SEC14p sc with membrane fractions containing the Golgi
integral membrane protein KEX2p; and (b) the coincidence
of SEC14p sc and KEX2p localization as judged by double
label immunofluorescence experiments (Cleves et al.,
1991b). KEX2p is a protease that is involved in the proteo-
lytic processing of specific prohormone precursors at sites
of dibasic residues (Fuller et al., 1989). Since the phe-
notypic consequences for loss of PI/PC-TP function are
very different in Y. lipolytica as opposed to S. cerevisiae,
we wished to determine the intracellular distribution of
SEC14p sc.

Treatment of fixed wild-type Y. lipolytica cells with a pri-
mary rabbit anti-SEC14p sc serum and FITC-conjugated
donkey anti-rabbit secondary antibodies yielded a bright
punctate staining pattern against a slight background of
diffuse cytoplasmic staining in all cells analyzed (Fig. 7).
This profile was judged to be SEC14p sc specific since: (a)
exclusion of the anti-SEC14p sc antibodies from the staining
regimen; or (b) use of fixed secl4 m::Δ1::LEU2 m cells in
the complete staining regimen, led to a complete loss of FITC
staining (not shown). Moreover, these SEC14p sc positive
structures failed to colocalize with mitochondria or the nu-
cleus as judged by comparing the FITC and DAPI staining
profiles. We did note, however, that SEC14p sc positive
structures frequently exhibited some clustering in a perinu-
clear region of the cell (Fig. 7). We estimate that at least 85%
of the several hundred cells analyzed exhibited this sort of
perinuclear clustering of SEC14p sc positive structures.

To determine whether SEC14p sc is associated with Y.
lipolytica Golgi bodies, we attempted to identify Y. lipo-
lytica Golgi bodies, using a primary mouse anti-KEX2p antisa-
ume and appropriate Texas red–conjugated antibodies (see
Materials and Methods). The rationale for using these pri-
mary KEX2p antibodies was that the Y. lipolytica XPR6 gene
product exhibits several properties that identify it as a
KEX2p homolog. First, the XPR6 gene of Y. lipolytica en-
codes an endoprotease that has been implicated in KEX2p-
like proteolytic processing, at dibasic residues, of the al-
kaline extracellular protease precursor during its transit
through a late Golgi compartment (Matoba et al., 1988;
Matoba and Ogrydziak, 1989). Second, the XPR6p primary
sequence is inferred to share significant homology with that
of KEX2p (Ogrydziak, D., personal communication). Fi-
ally, immunoblotting of Y. lipolytica lysates with anti-
KEX2p serum reveals a single KEX2p-immunoreactive spe-
cies (110 kD) that is not observed when lysates prepared
from Δxpr6 strains are similarly probed (not shown). Thus,
we used XPR6p as a putative Y. lipolytica Golgi marker, and the
XPR6p staining profile is shown in Fig. 7. As in the case
of SEC14p sc, the XPR6p staining profile was punctate in
character with numerous XPR6p positive structures per cell.
This staining pattern was very similar to that observed for
SEC14p sc, even with respect to the clustering of XPR6p-
containing structures in perinuclear regions. Again, either
exclusion of XPR6p antibodies or use of Δxpr6 strains of Y.
lipolytica in the complete staining regimen precluded detec-
tion of Texas red signal (not shown).

Superimposition of the SEC14p sc and XPR6p profiles by
digital image processing revealed a high degree of colocal-
Figure 6. sec4Δ strains fail to execute the transition from yeast to mycelium. Wild-type (MCL28) and sec4Δ::URA3Δ (MCL30) strains were grown on either solid (a) or liquid (b) YPD medium at 28°C. (a) Colony morphology after 36 h of growth on solid YPD medium is typified by the rough phenotype for the wild-type strain (left) and the smooth phenotype for the sec4Δ::URA3Δ strain (right). (b) Cell morphology after 2 h (top) or 24 h (bottom) of growth in liquid YPD medium at 28°C. The mycelial form taken by the wild-type strain (MCL28, left) is in sharp contrast to the pseudomycelial form adopted by the sec4Δ::URA3Δ strain (MCL30, right).
Figure 7. SEC14p\textsuperscript{yl} colocalizes with the presumptive Golgi marker XPR6p. The wild-type \textit{Y. lipolytica} strain POla was grown to early logarithmic phase in YNB medium, cells were fixed in 3.7\% formaldehyde, and prepared for immunofluorescence microscopy as described in Materials and Methods. The XPR6p profile was revealed by sequential incubation of cells with a primary mouse polyclonal antibody raised against the \textit{S. cerevisiae} KEX2p, a secondary polyclonal sheep anti-mouse antiserum, and Texas red–conjugated donkey anti-sheep antibodies (top left). The SEC14p\textsuperscript{yl} profile was revealed by serial incubation of these same cells with a primary rabbit antiserum directed against the \textit{S. cerevisiae} SEC14p and secondary FITC-conjugated donkey anti-rabbit antibodies (top right). Areas of colocalization appear yellow in the computer-generated composite image (bottom left). A Nomarski image is shown in the bottom right panel. Both the XPR6p and SEC14p\textsuperscript{yl} signals were completely abolished by either the exclusion of the corresponding primary antibodies from the staining regimen, or by introduction of \textit{Δxpr6} and \textit{Δsec14} alleles into the \textit{Y. lipolytica} strain to be tested.

ization between these two markers (Fig. 7). Points of complete coincidence between XPR6p and SEC14p\textsuperscript{yl} staining appear as yellow areas, regions where stained structures are in immediate proximity appear orange, and points of non-coincidence exhibit a color that corresponds to the fluorophore being visualized (FITC stains green). Examination of approximately 200 cells led us to estimate that greater than 60\% of the structures that stained positive for either XPR6p or SEC14p\textsuperscript{yl} stained positive for both. Extensive coincidence of staining was especially apparent in the perinuclear region. These data demonstrate that XPR6p and SEC14p\textsuperscript{yl} exhibit a high, but not absolute, degree of colocalization to a disperse cytoplasmic organelle of the \textit{Y. lipolytica} secretory pathway, likely the Golgi complex.

\textit{sec14}\textsuperscript{yl} Mutants Exhibit Wild-type Secretory Pathway Function

The viability of \textit{Δsec14} strains indicated that SEC14p\textsuperscript{yl} was largely dispensable for secretory functions in \textit{Y. lipolytica}, the localization of SEC14p\textsuperscript{yl} to what are presumed to be \textit{Y. lipolytica} Golgi bodies notwithstanding. To investigate this issue further, we compared the kinetics of secretion of a periplasmic acid phosphatase and an AEP in wild-type and \textit{sec14}\textsuperscript{yl} mutant stains. The \textit{sec14}::\textit{URA3} allele interrupts SEC14p\textsuperscript{yl} after residue 163 (Fig. 1 and 2). As interruption of SEC14p\textsuperscript{sc} at residue 234 represents a null mutation (i.e., \textit{sec14}\textsuperscript{Δ}::\textit{HIS3}; Bankaitis et al., 1989), we believe that \textit{sed4}::\textit{URA3} also represents a null mutation.

AP is a highly glycosylated enzyme whose synthesis is derepressed upon imposition of phosphate limitation on cells grown on minimal medium (Lopez and Dominguez, 1988). On such a medium, both wild-type and \textit{sec14}::\textit{URA3} strains remained in the yeast form and grew at identical rates (Fig. 8 A). Analysis of extracellular AP activity at various times post-induction revealed that the kinetics of appearance and the amounts of AP activity at the cell surface were very similar in both wild-type and \textit{sec14}::\textit{URA3} strains (Fig. 8 B). In contrast to AP, AEP is induced when cells are grown in YPD medium; a condition where wild-type cells undergo differentiation from yeast cells to hyphae but \textit{sec14}::\textit{URA3} strains cannot (see above). AEP is synthesized as a 55-kD
precursor (pAEP) which subsequently undergoes a complex maturation to finally yield the mature 32-kD secreted product (mAEP; Matoba et al., 1988). We used a pulse–chain regimen followed by immunoprecipitation to sensitively monitor the biogenesis of AEP in wild-type and sec14Δ::URA3Δ strains. The rate of conversion of pAEP to mAEP, indicative of transit of pAEP from the ER to a late Golgi compartment (Matoba and Ogrydziak, 1989), was quite rapid in wild-type cells (Fig. 8C). Some 60% of the pAEP had been matured by 1.5 min of chase and maturation was complete by 6 min of chase. Similar kinetics of pAEP maturation were measured for the sec14Δ::URA3Δ strain. These data indicated that AEP transit through the early stages of the Y. lipolytica secretory pathway was not affected in sec14Δ::URA3Δ strains. The kinetics of mAEP transit from the Golgi to the cell surface, and subsequently into the extracellular medium, were estimated by monitoring the rate of pulse-radiolabeled mAEP appearance in the growth medium as a function of the time of chase. Those data are also presented in Fig. 8C. In both wild-type and sec14Δ::URA3Δ strains, small amounts of mAEP were detected in the medium at the earliest chase point taken (1.5 min). By 6 min of chase, secretion of mAEP into the medium appeared to be complete for both strains tested. The collective data demonstrate that the efficiency and rate of protein transport through the Y. lipolytica secretory pathway was not detectably impaired by SEC14pYL dysfunction.

Discussion

The study of SEC14pSC function in S. cerevisiae has established a paradigm for PI/PC-TP function in vivo. Penetrating clues as to the mechanism of SEC14p function in vivo have been forthcoming from a genetic analysis of mutants that no longer require SEC14pSC for Golgi secretory function and cell viability (Cleves et al., 1989; Cleves et al., 1991b). These studies revealed that inactivation of a particular one of the two pathways available for PC biosynthesis in S. cerevisiae effects bypass of the normally essential SEC14pSC requirement. This PC biosynthetic pathway of interest, the CDP-choline pathway, consists of three reactions that result in the incorporation of free choline into PC via a cytidine-based mechanism (Kennedy and Weiss, 1956). The finding that the cellular requirement for SEC14p is obviated by inactivation of a specific avenue for PC biosynthesis has led to the proposal that SEC14pSC is involved in controlling the phospholipid composition of yeast Golgi membranes: a function that is consistent with what one might expect of a genuine PL-TP (Cleves et al., 1991a,b). However, while subsequent biochemical analyses indicate that SEC14pSC does indeed control the PC content of yeast Golgi membranes, thereby confirming a basic tenet of that hypothesis, those same biochemical studies have raised the possibility that such control of Golgi PC content may well use a mechanism that does not involve genuine lipid transfer (McGee et al., 1994). Rather, the idea that SEC14pSC acts as a Golgi phospholipid sensor through which a Golgi-localized aspect of the CDP-choline pathway is regulated must also be considered (McGee et al., 1994). Yet, the in vitro PI/PC-TP activity of SEC14pSC is believed to somehow reflect an essential functional property of SEC14pSC as evidenced by the ability of a mammalian PI/PC-TP, which exhibits no primary se-

Figure 8. Protein secretion in wild-type and sec14Δ strains. Isogenic Y. lipolytica strains were monitored for their growth (a), periplasmic phosphatase activity (b), and secretion of alkaline extracellular protease (c). The two strains, MCL28 (SEC14Δ) and MCL30 (sec14Δ::URA3Δ), were cultured in low phosphate medium at 28°C (a and b) or in YPD medium (c). At the indicated times, culture samples were analyzed for OD600 (a) and periplasmic phosphatase activity (b). 1 U of phosphatase activity is defined as the amount of enzyme releasing 1 nmole of p-nitrophenol in 1 min at 30°C. To monitor protease secretion (c), cells were pulse-radiolabeled for 2 min with [3H]leucine (3,000 Ci/m mole, L-[4,5-3H]-leucine; Amersham) and subjected to a chase with cold leucine (1% final concentration). Samples were taken at the indicated times post-chase, and cell-associated AEP was immunoprecipitated from clarified cell lysates with anti-AEP serum, immunoprecipitates were resolved by SDS-PAGE, and visualized after fluorography for 5 d at -80°C (top). Total culture supernatant proteins collected from the corresponding samples were precipitated with TCA, the TCA precipitates were resolved by SDS-PAGE, and visualized after overnight fluorography at -80°C. Positions of the 55-kD AEP precursor, and the 30-kD mature AEP, are indicated at left.
quency similarity to SEC14pSC, to complement the S. cerevisiae sec14-1 mutation (for a discussion see Skinner et al., 1993). Moreover, although it is not yet precisely clear as to why SEC14pSC-mediated control of Golgi membrane PC content is an essential activity in S. cerevisiae, it has recently been suggested that SEC14pSC function might be a prerequisite for the appropriate turnover of inositol phospholipids in yeast Golgi membranes so that Golgi secretory function can be stimulated (Whitters et al., 1993).

One of the many important questions that has arisen since the establishment of SEC14pSC as a paradigm for the in vivo function of a PL-TP is how generally applicable are the basic features of the SEC14pSC paradigm to the in vivo function of other PL-TPs, or even other PI/PC-TPs. An examination of this issue requires the isolation and characterization of sec14 mutants in organisms unrelated to S. cerevisiae. The demonstration that the widely divergent yeasts K. lactis and S. pombe each exhibit a polypeptide with high primary sequence similarity and functional homology to SEC14pSC, coupled with the ability of expression of a mammalian PI/PC-TP to correct sec14-1" growth and secretory defects in S. cerevisiae, have led to a proposal that the biological function of PI/PC-TPs might be conserved across wide evolutionary distances (Bankaitis et al., 1989; Salama et al., 1990; Cleves et al., 1991; Skinner et al., 1993). A rigorous test of this possibility requires the availability of sec14 mutants in other organisms. For this reason, we characterized SEC14pYL function and localization. The Y. lipolytica system was well suited for these studies due to: (a) its facility for genetic manipulation; (b) the vast evolutionary distance that separates this dimorphic yeast from S. cerevisiae. The demonstration that the widely divergent yeasts K. lactis and S. pombe each exhibit a polypeptide with high primary sequence similarity to SEC14pSC, to complement the S. cerevisiae and S. pombe (Franzusoff et al., 1991; Cleves et al., 1991b; Redding et al., 1991; Chappell and Warren, 1989; Preuss et al., 1992). These data lead us to conclude that, in direct analogy to the localization of SEC14pSC in S. cerevisiae, SEC14pYL is associated, at least in part, with the Y lipolytica Golgi complex. This conclusion must be considered tentative, however, as the suitability of XPR6p as a Yarrowia Golgi marker is based largely on circumstantial evidence (i.e., its homology to the S. cerevisiae Golgi marker KEX2p; see above) and is not yet rooted in evidence obtained from more direct subcellular localization experiments. Nonetheless, the association of SEC14pYL with compartments of the Yarrowia secretory pathway is probable.

A surprising distinction between SEC14pSC and SEC14pYL function in vivo was forthcoming from gene disruption experiments. Whereas SEC14pSC is essential for the viability of wild-type S. cerevisiae (Bankaitis et al., 1989), SEC14pYL clearly was not required for the viability of Y. lipolytica under conditions of vegetative growth or germination from spores. Moreover, SEC14pYL dysfunction was of no consequence to secretory pathway function in Yarrowia, as determined by comparing the efficiency and kinetics of AP and AEP secretion in wild-type and sec14" strains (Fig. 8, b and c). The nonessentiality of SEC14pYL is not readily explained by the presence of some redundant function as evidenced by our inability to detect SEC14pYL homologs by nucleic acid hybridization experiments or immunoblotting experiments with anti-SEC14pSC serum (Fig. 3, a and b). Moreover, phospholipid transfer assays using lysates prepared from wild-type and sec14" mutant strains of Y. lipolytica identified SEC14pYL as the major, and perhaps the only, Yarrowia PI/PC-TP (Fig. 4). Thus, the available evidence leads us to conclude that SEC14pYL (and therefore PI/PC-TP activity) is genuinely nonessential for Y. lipolytica viability. Our finding that Y. lipolytica has an active CDP-choline pathway for PC biosynthesis also excludes from further consideration the trivial possibility that the nonessentiality of SEC14pYL could result from Yarrowia being naturally incompetent for PC synthesis via this pathway (Fig. 5); a mechanism by which the normally essential SEC14pSC function can be bypassed in S. cerevisiae (Cleves et al., 1991b). These data further reinforce the distinction between the in vivo functions of SEC14pSC and SEC14pYL. Thus, irrespective of the significant aspects of functional similarity shared by SEC14pSC and SEC14pYL, these PI/PC-TPs are involved in controlling distinct physiological processes in their respective host organisms. Finally, the finding that SEC14pYL is a genuinely nonessential function in Y. lipolytica provides yet another clear example where a PL-TP does not play an essential role in the recycling of bulk

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membrane lipid from the late stages of the secretory pathway back to the ER (Wieland et al., 1987; Rothman, 1990; Cleves et al., 1994a,b).

While SEC14p<sup>vt</sup> function is not essential for Yarrowia viability, our genetic data demonstrated a requirement for SEC14p<sup>vt</sup> in the dimorphic transition of the yeast form into the mycelial form. Yarrowia secl<sup>vt</sup> mutants were uniformly incapable of forming hyphae, even under growth conditions that favor mycelium formation in wild-type cells, and therefore exhibited a smooth colony phenotype, as opposed to the rough colony morphology typical of wild-type strains (Fig. 6, a and b). This differentiation from yeast to mycelium is a very poorly understood developmental program that involves a dramatic reorientation with respect to cell morphology (avoid to filamentous), mode of culture growth (exponential to linear), and mechanism of cell division (bud- ding mode to septation). The inability of secl<sup>vt</sup> strains to assume a true mycelial form when the culture achieves stationary phase (Fig. 6 b), indicates a role for SEC14p<sup>vt</sup> in promoting at least one of these transition steps.

How might SEC14p<sup>vt</sup> function in promoting the dimorphic transition event? There is evidence to suggest that execution of the dimorphic transition program in another yeast, Candida tropicalis, might require enhanced turnover of phosphatidylinositol (Uejima et al., 1987). Additional support for a role for PI metabolism in this differentiation event is provided by the observation that addition of free inositol to C. tropicalis inhibits the transition of yeast cells to mycelium (Tani et al., 1979), and the demonstration of an involvement for the Ras signal transduction pathway in controlling a less well-developed version of the dimorphic transition event in S. cerevisiae (Gimeno et al., 1992). Indeed, one can easily imagine that a PI/PC-TP could somehow play a role in controlling PI metabolism via its PI-transfer activity (perhaps by acting to present inositol phospholipid to an appropriate phospholipase) and therefore exert a significant influence on a cellular process that uses PI turnover for its execution. Thus, the clear elements of conservation between these PI/PC-TPs notwithstanding, it is possible that SEC14p<sup>vt</sup> and SEC14p<sup>sc</sup> may play mechanistically divergent roles in their respective organisms. However, there is in vivo evidence to suggest that, although the primary function of SEC14p<sup>sc</sup> appears to be a regulatory activity directed at the control of Golgi membrane PT content (McGee et al., 1994), the ultimate consequence of SEC14p<sup>sc</sup> function may be to either generate the appropriate PL composition in the yeast Golgi complex so that the requisite level of PI turnover can be sustained to drive Golgi secretory processes (Cleves et al., 1994a; Whitters et al., 1993), or to maintain an appropriate diacylglycerol pool in yeast Golgi membranes (McGee et al., 1994). These concepts are based largely on the behavior of sac1 mutants that exhibit: (a) the ability to suppress mutations in the actin structural gene of yeast in an allele-specific manner (Novick et al., 1988); (b) the ability to suppress secl<sup>vt</sup> null mutations (Cleves et al., 1989); and (c) a novel inositol auxotrophy that is not related to an inositol biosynthetic difficulty, but to an elevated inositol requirement for growth (Whitters et al., 1993). Thus, the possibility also remains that SEC14p<sup>sc</sup> and SEC14p<sup>vt</sup> may play mechanistically similar regulatory roles in their respective host organisms in vivo, but that the regulatory function of these proteins is simply coupled to downstream circuits that have different target processes. In that regard, it will be of great interest to determine what role the Y. lipolytica Golgi complex plays in promoting the dimorphic transition developmental program.

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