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 *SEC14*^{SC} gene encodes the phosphatidylinositol/phosphatidylcholine transfer protein (PI/PC-TP) of *Saccharomyces cerevisiae*. The *SEC14*^{SC} gene product (SEC14p^{SC}) 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 *SEC14*^{YL}, the structural gene for the PI/PC-TP of the dimorphic yeast *Yarrowia lipolytica*. *SEC14*^{YL} encodes a primary translation product (SEC14p^{YL}) that is predicted to be a 497-residue polypeptide of which the amino-terminal 300 residues are highly homologous to the entire SEC14p^{SC}, 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 SEC14p^{SC}, SEC14p^{YL} localizes to punctate cytoplasmic structures in *Y. lipolytica* that likely represent Golgi bodies. However, SEC14p^{YL} is neither required for the viability of *Y. lipolytica* nor is it required for secretory pathway function in this organism. This nonessentiality of SEC14p^{YL} 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 Δ*sec14*^{YL} strains are devoid of measurable PI/PC-TP activity in vitro. Phenotypic analyses demonstrate that SEC14p^{YL} 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, Δ*sec14*^{YL} mutants form aberrant pseudomycelial structures as cells enter stationary growth phase. The collective data indicate a role for SEC14p^{YL} 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 organellar 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;

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Pagano, 1990; Voelker, 1991): (*a*) transfer of lipid via conventional transport vesicles that are also dedicated to protein transport through the secretory pathway (e.g., glycolipid transport to the plasma membrane); (*b*) transfer of lipid by vesicular carriers that do not participate in protein transport through the secretory pathway (e.g., transport of cholesterol from the ER to the plasma membrane); (*c*) collision-mediated transfer of lipid between organelles (e.g., transport of phosphatidylserine from the ER to mitochondria); and (*d*) transfer of lipids as monomers through the cytosol. The phospholipid transfer proteins (PL-TPs)[1] have long been considered attractive candidates for executing intracellular lipid traffic of the sort exemplified by mechanism (iv). These PL-TPs are cytosolic proteins that have the capability of acting as diffusible carriers that transport lipid monomers between membrane bilayers in vitro, and are distinguished on the basis of the phospholipid headgroup specificities they exhibit in the in vitro transfer reaction (Wirtz, 1991; Cleves et al., 1991*a*).

Although PL-TPs have been extensively characterized with respect to the biochemistry of their catalytic properties, an appreciation of their in vivo role has been elusive. The finding that the *Saccharomyces cerevisiae* SEC14 gene product (SEC14p^{SC}) is a phosphatidylinositol/phosphatidylcholine transfer protein (PI/PC-TP) whose function is essential for both yeast Golgi secretory function and for cell viability, provided the initial insight into the biological function of a PL-TP (Bankaitis et al., 1989, 1990). As such, the analysis of SEC14p^{SC} function has set the paradigm for PL-TP function in vivo. A considerable body of evidence indicates that SEC14p^{SC} plays an essential role in controlling the PC content of yeast Golgi membranes (Cleves et al., 1991; McGee et al., 1994). Although the precise mechanism by which SEC14p^{SC} achieves such a control of Golgi phospholipid composition has not yet been resolved, current data raise the issue of whether SEC14p^{SC} functions as a genuine PI/PC-TP in vivo (for a discussion see McGee et al., 1994). Since SEC14p^{SC} presently defines the sole in vivo model for PL-TP function, the question of how generally applicable the SEC14p^{SC} paradigm is to the in vivo function of other PL-TPs, or even other PI/PC-TPs, is an important one. The widely divergent yeasts *Kluyveromyces lactis* and *Schizosaccharomyces pombe* exhibit polypeptides both structurally and functionally homologous to SEC14p^{SC} as judged by: (*a*) comparison of primary sequences inferred from nucleotide sequence analysis of the respective genes; and (*b*) the ability of these heterologous SEC14ps to fulfill all essential SEC14p^{SC} functions when expressed in *S. cerevisiae* (Bankaitis et al., 1989; Salama et al., 1990; Skinner, H. B., and V. A. Bankaitis, manuscript in preparation). These findings have raised the possibility that the biological function of PI/PC-TPs might be conserved across wide evolutionary distances. A rigorous test of this possibility requires the availability of *sec14* mutants in other organisms.

In this report, we describe the isolation and characterization of *SEC14*^{YL}, the structural gene for the major PI/PC-TP of the dimorphic yeast *Yarrowia lipolytica*. This yeast is widely diverged from both *S. cerevisiae* and *S. pombe* (Barns et al., 1991) and is typified by two distinct developmental forms, the yeast and the mycelial forms, whose predominance can be controlled at the level of the growth medium (Rodriguez and Dominguez, 1984). Our data indicate a considerable level of functional homology between SEC14p^{SC} and SEC14p^{YL} as evidenced by the ability of the latter to efficiently substitute for the essential function of the former *S. cerevisiae*. Also, in a manner entirely analogous to the SEC14p^{SC} paradigm, we find that SEC14p^{YL} is a PI/PC-TP that localizes to what are likely to be *Y. lipolytica* Golgi bodies. However, in stark contrast to the case of SEC14p^{SC} in *S. cerevisiae*, SEC14p^{YL} is neither required for the cellular viability of *Y. lipolytica* nor is it required for efficient secretory pathway function. Furthermore, we provide strong evidence to indicate that the nonessentiality of SEC14p^{YL} for growth and secretion in *Y. lipolytica* is not attributable to the presence of a functionally redundant PI/PC-TP activity. Finally, the only phenotypic consequence of SEC14p^{YL} dysfunction we have discerned is the inability of $\Delta sec14^{YL}$ *Y. lipolytica* strains to undergo the dramatic yeast-mycelial transition that is typical of this species. The collective data demonstrate that, irrespective of the functional relatedness of SEC14p^{SC} and SEC14p^{YL}, these PI/PC-TPs are involved in controlling distinct physiological processes in their respective host organisms.

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: E122, 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-P_i medium, respectively (Nicaud et al., 1989; Lopez and Dominguez, 1988). *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 allele 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 μm 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 *SEC14*^{YL} by PCR were 5′-GAGCGAATGCTGAAGAACCTGGTCTGGGAGTACGA-3′ (primer b) and 5′-GTAGAACTTTCCCATTCGCTCGGGGTAGTAGTTCTG-3′ (primer c). These primers were designed taking into account the *Y. lipolytica* codon bias (Nicaud et al., 1989) and correspond to codons specifying residues 141-152 and 202-213 of the SEC14p^{SC} and SEC14p^{KL} primary sequence, respectively, which are conserved (Salama et al., 1990). For amplification

1. *Abbreviations used in this paper*: AEP, alkaline extracellular protease; BHT, butylated hydroxytoluene; PC-TP, phosphatidylcholine transfer protein; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL-TP, phospholipid transfer proteins; PS, phosphatidylserine.

Table I. Plasmids and Strains Used

Plasmids

Name	Description	Source or reference
pCTY11	*S. cerevisiae* YEp vector carrying *LEU2*, *ADE3*, and *SEC14*	V. Bankaitis
pFL61	*S. cerevisiae* expression vector based on *PGK* promoter and terminator, carrying *URA3*	Minet et al., 1992
pINA62	5.6-kb SalI fragment carrying $LEU2^{YL}$ in pBR322	Gaillardin and Ribet, 1987
pINA237	pBR322 carrying LEU^{YL} and *ARS-CEN18*	Fournier et al., 1993
pINA300′	1.5-kb SalI fragment carrying $URA3^{YL}$ in pBR322	This laboratory
pINA476	*XPR2* terminator and $LEU2^{YL}$ gene in pBR322	Tharaud et al., 1992
pINA540	7.2-kb Sau3A fragment carrying $SEC14^{YL}$ in pINA62	This work
pINA543	3.6-kb HindIII-Sau3A fragment carrying $SEC14^{YL}$ in pINA62	This work
pINA651	3.6-kb HindIII-Sau3A fragment carrying $SEC14^{YL}$ in pBR322	This work
pINA652	1.5-kb SalI fragment carrying $URA3^{YL}$ from pINA300′ into pINA651 (*sec14::URA3*)	This work
pINA653	3.6-kb HindIII-Sau3A fragment carrying $SEC14^{YL}$ in pINA 237	This work
pINA656	pINA300′ with filled-in EcoRI site	This laboratory
pINA657	0.48-kb NruI-XhoI internal deletion of $SEC14^{YL}$ in pINA656 (*sec14Δ1*)	This work
pINA926	1.6-kb cDNA of $SEC14^{YL}$ in pFL61	This work
pINA929	2.7-kb StuI-BalI deletion of $SEC14^{YL}$ in pBR322	This work
pINA930	3.1 kb *Pst*I fragment carrying $LEU2^{YL}$ in pINA929 (*sec14Δ2:LEU2*)	This work
pRE510	*S. cerevisiae SEC14* cDNA in pTZ18	D. Malehorn and Bankaitis

Strain	Genotype	
S. cerevisiae		
CTY1-1A	*MATa, ura3-52, Δhis3-200, lys2-801, sec14-1*	McGee and Bankaitis
CTY558	*MATa,ade2, ade3, leu2, Δhis3-200, ura3-52, sec14Δ1::HIS3*/pCTY11	Bankaitis
MCL35	*MATa, ura3-52, Δhis3-200, lys2-801, sec14-1*/pINA926	Transformation of CTY1-1A
Y. lipolytica		
W29	*MatA*	Wild type (our collection)
E122	*MatA, lys11-23, Δleu2-270, Δleu2-270, Δura3-302*	Fabre et al., 1992
PO1a	*MatA, Δleu2-270, Δura3-302*	W29 derivative, Tharaud et al., 1992
JM12	*MatB, leu2-35, lys5-12, ura3-18*	Nicaud et al., 1989
MCL8	*MatA/MatB, +/Δleu2-270, +/lys11-23, +/his-1, Δura3-302/Δura3-302, SEC14/sec14::URA3*	This work
MCL9	*MatA, lys11-23, Δleu2-270, Δura3-302/pINA653*	E122+pINA653
MCL9D	*MatA, lys11-23, Δleu2-270, Δura3-302, sec14::URA3*/pINA653	MCL9 + HindIII-SalI fragment of pINA652
MCL12	*MatA, lys11-23, Δleu2-270, Δura3-302, sec14Δ1*	E122 derivative, see Material and Methods
MCL25	*MatA, lys11-23, Δleu2-270, Δura3-302, sec14::URA3*	MCL9 segregant
MCL27	*MatA, Δleu2-270, Δura3-302, sec14::URA3*	PO1a+HindIII-SalI fragment of pINA652
MCL28	*MatB, lys5-12, ura3-18*	JM12+NotI-digested pINA62
MCL29	*MatB, leu2-35, lys5-12, ura3-18, sec14::URA3*	JM12+HindIII-SalI fragment of pINA652
MCL30	*MatB, lys5-12, ura3-18, sec14::URA3*	MCL29+NotI-digested pINA62
MCL41	*MatA, Δleu2-270, Δura3-302, sec14Δ2::LEU2*	PO1a+SphI fragment of pINA930

of *Y. lipolytica* DNA, the PCR reactions contained 150 ng of DNA, and 25 pmoles 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™ 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 $SEC14^{YL}$ 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 $sec14^{YL}$ alleles were constructed. First, pINA652 (see Fig. 2 *b*) carries a simple $sec14^{YL}$*::URA3* disruption allele. This plasmid was constructed by inserting the $URA3^{YL}$ gene, as a 1.5-kb SalI restriction fragment (from pINA300′; see Table 1), into the unique XhoI site present in pINA651, thus interrupting the $SEC14p^{YL}$ coding sequence 162 codons downstream of the initiator codon. Second, the $sec14^{YL}\Delta1$ allele represents a 0.4-kb deletion bounded by the NruI and XhoI sites of $SEC14^{YL}$, 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)-SphI fragments of pINA651 into pINA300′ cleaved with ClaI and SphI. Third, a complete deletion of $SEC14^{YL}$ (designated $sec14^{YL}\Delta2$) was constructed by digesting pINA651 with StuI (cleaves at nucleotide 37 as designated in Fig. 1) and BalI (nucleotide 2759; see Fig. 1), and religation of the cleaved plasmid to yield pINA929. Subsequent insertion of a 3.1-kb PstI fragment carrying $LEU2^{YL}$ into the PstI site of pINA929 yielded the $sec14^{YL}\Delta2$*::LEU2* plasmid pINA930 (see Fig. 2 *d*). Replacement of the genomic $SEC14^{YL}$ gene with the $sec14^{YL}\Delta1$ allele was via a two step gene substitution protocol (Boecke et al., 1987). Plasmids were targeted to the

genomic *SEC14*^{YL} 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*^{YL} sequences flanking the integrated vector. The recombinants in which the desired gene replacement event had occurred were identified by Southern analysis (Table I).

Immunochemical 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^{SC} serum raised against a TrpE-SEC14p^{SC} fusion protein (Bankaitis et al., 1989) was used to visualize SEC14p^{YL} 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^{SC} serum was used at a 1:500 dilution and the immunoblots were developed with an alkaline phosphatase-conjugated secondary antibody obtained from Promega Biotec (Madison, WI) (1: 10,000 dilution).

Immunofluorescence experiments were performed as previously described (Pringle et al., 1989; Cleves et al., 1991*b*). *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 FITC-conjugated donkey anti-rabbit antibodies (Jackson ImmunoResearch, West Grove, PA) for 2 h at a concentration of 30 μg/ml each. After a last 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 SIT video camera (Dage-MTI Inc., Wabash, WI) 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, Montvale, 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, 3 mM NaN_3. The resulting suspension was dialyzed against 20 vol of the same buffer. Finally, the dialysate was adjusted to pH 5.1, recentrifuged 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 [^{32}P]orthophosphate ($^{32}P_i$; 10 μCi/ml) for 30 min at 25°C with shaking. For steady-state $^{32}P_i$-labeling experiments, yeast were grown in synthetic complete medium overnight, subcultured, and then presented with $^{32}P_i$ (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 $CHCl_3/CH_3OH$/butylated hydroxytoluene (BHT) (2:1: 0.0005%), dried under N_2 gas, and resuspended in $CHCl_3/CH_3OH$/BHT. Radiolabeled phospholipids were resolved by two-dimensional chromatography using Whatman SG81 paper (Steiner and Lester, 1972). First dimension solvent was $CHCl_3/CH_3OH/NH_4OH/H_2O$ (22: 9: 1: 0.26) and second dimension solvent was $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (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

Periplasmic acid phosphatase was measured as described by Lopez and Dominguez (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 SEC14^{YL} *Gene*

We used a PCR strategy to recover genomic *SEC14*^{YL} clones and a complementation strategy to recover cDNA clones of *SEC14*^{YL} (see Materials and Methods). A 219-bp genomic 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^{SC} 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 *SEC14*^{YL} gene was subcloned into pBR322 to generate pINA651 (Table I).

The *Y. lipolytica* cDNA expression library was transformed into the *ura3-52*, *sec14-1*^{ts} *S. cerevisiae* strain CTY1-1A and Ura$^+$ transformants were selected at 25°C, a permissive temperature for *sec14-1*^{ts} yeast strains. A total of approximately 20,000 Ura$^+$ transformants were screened for growth at a temperature restrictive for *sec14-1*^{ts} 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 *sec14-1*^{ts} complementing cDNA indicated that the two plasmids carried identical cDNA in-

serts 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 I). Moreover, the restriction maps of the cDNA clones corresponded closely to that deduced for the candidate genomic *SEC14*^{YL} clone identified by in situ hybridization.

These data suggested that the *SEC14*^{YL} genomic and cDNA clones identified a *Y. lipolytica* homolog of *SEC14p*^{SC}. 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 inheritance of *sec14*^{SC} null alleles by haploid *S. cerevisiae* strains (Bankaitis et al., 1989). *S. cerevisiae* strain CTY558 (*ade2, ade3, leu2, ura3, sec14Δ1::HIS3*) carries plasmid pCTY11, a YEp(*LEU2,ADE3*) vector where the *SEC14*^{SC} gene is under the control of an attenuated *SEC14*^{SC} promoter and drives the synthesis of SEC14p^{SC} in yeast at a rate similar to that normally sustained by the genomic *SEC14*^{SC} locus (Table I; Whitters et al., 1993). Strain CTY558 is absolutely dependent on pCTY11 for viability as this plasmid complements the haploid lethal *sec14Δ1::HIS3* allele. Moreover, CTY558 forms uniformly red colonies on all media, a characteristic phenotype of *ade2* strains of *S. cerevisiae*, whereas *ade2, ade3* double mutants are white. Thus, loss of pCTY11 from CTY558 can be visually scored by the appearance of white sectors or white colonies in a background of otherwise red colonies. We transformed strain CTY558 with the cDNA clone pINA926 and selected for Ura$^+$ Leu$^+$ transformants, thereby demanding the presence of both pCTY11 and pINA926 in the transformants. The desired transformants were picked and streaked for isolation on YPD medium. We found the CTY558/pINA926 transformants to yield white sectors at a very high frequency. All white segregants were Leu$^-$ and Ura$^+$, signifying loss of pCTY11 and retention of pINA926, respectively. The latter point was confirmed by recovering and characterizing the resident plasmids of the pCTY11 segregants (not shown). Finally, as expected, we were no longer able to detect curing of pINA926 from the pCTY11 segregants on any media; indicating that these segregants were now dependent on pINA926 for viability. These collective data demonstrate that pINA926 was able to substitute for pCTY11 in sustaining viability of the *sec14Δ1::HIS3* strain, and suggested we had recovered cDNA and genomic clones encoding the SEC14p^{YL}.

Nucleotide Sequence of Genomic SEC14^{YL} *Gene and SEC14*^{YL} *cDNA*

The sequences of both the *SEC14*^{YL} 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 *SEC14*^{YL} clone. To further clarify the physical organization of *SEC14*^{YL} we compared the genomic and cDNA sequences. The *SEC14*^{YL} 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 *SEC14*^{YL} 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 *SEC14*^{YL} 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 *SEC14*^{YL} 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 *SEC14*^{YL}. The 5′-splice sites of both introns corresponded to a GTGAGTPu motif which diverges from the consensus GTATGT 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 *SEC14*^{YL} 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 *SEC14*^{YL} 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 *SEC14*^{YL} nucleotide sequence predicted a gene product of 497 residues (58 kD), a prediction confirmed by identification of the SEC14p^{YL} in immunoblots of *Y. lipolytica* cell-free extracts (see below). Thus, SEC14p^{YL} 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 SEC14p^{YL} primary sequence with those of SEC14p^{SC} and SEC14p^{KL} revealed that the first 300 residues of SEC14p^{YL} shared 65 and 65.8% identities, respectively, with the full-length primary sequences of these SEC14p species. The carboxy-terminal 197 SEC14p^{YL} residues have no counterpart in SEC14p^{SC} and SEC14p^{KL} primary sequences and share no significant similarity with protein sequences currently entered in protein data bases. One notable feature of the carboxy-terminal SEC14p^{YL} 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).

SEC14p^{YL} *Function Is Not Essential for the Viability of Y. lipolytica*

The *SEC14*^{SC} gene is essential for cell viability in *S.*

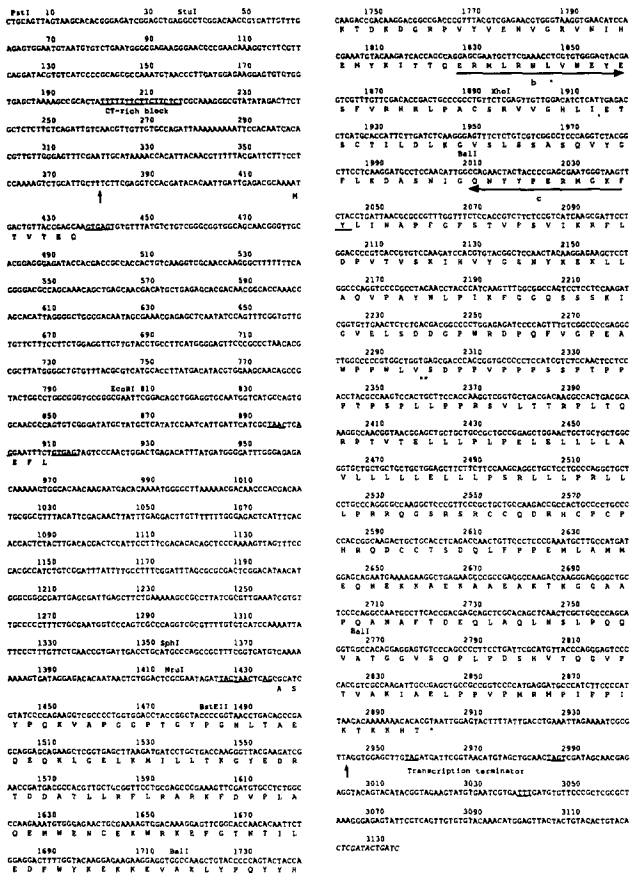

Figure 1. SEC14^{YL} sequence. The nucleotide sequence of the genomic PstI-Sau3a fragment is given, as is the inferred protein sequence (in one letter code). Consensus sequences for intron splicing and transcription termination are underlined, as are potential transcription initiation elements. Vertical arrows indicate the 5′- and 3′-boundaries of the cDNA clones. The positions of PCR primers b and c used for amplification of *SEC14^{YL}* 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^{YL}* sequence data are available from EMBL/GenBank/DDBJ under accession number L20972.

cerevisiae (Bankaitis et al., 1989), but it is not yet known what the *sec14^{KL}* and *sec14^{SP}* null phenotypes are in *K. lactis* and *S. pombe*, respectively. We used two different strategies to determine whether *SEC14^{YL}* was essential for the viability of *Y. lipolytica*. First, we used a plasmid segregation test to determine the essentiality of *SEC14^{YL}* for vegetative growth of *Y. lipolytica*. Strain MCL9 contains pINA653, a centromeric *SEC14^{YL},LEU2^{YL}* plasmid (Table I). The genomic *SEC14^{YL}* gene of MCL9 was replaced by the *sec14^{YL}::URA3^{YL}* allele (see Materials and Methods), and the expected disruption event was confirmed by Southern analysis. The *sec14^{YL}::URA3^{YL}*/pINA653 strains were then cultured on leucine-rich medium to relieve the nutritional selection pressure for pINA653, and we monitored the subsequent ability of these merodiploid strains to undergo spontaneous curing of pINA653. If the *sec14^{YL}::URA3^{YL}* allele represented a haploid-lethal mutation, the strains would experience an unrelenting selection pressure for retention of pINA653 and no spontaneous curing of this plasmid would be observed. If *sec14^{YL}::URA3^{YL}* were a nonlethal mutation, however, spontaneous curing of pINA653 should occur at a detectable frequency and Leu$^-$ segregants should appear. Surprisingly, Leu$^-$ segregants were readily obtained and exhibited normal growth rates on both minimal and YPD media, indicating that the *sec14^{YL}::URA3^{YL}* allele did not affect *Y. lipolytica* cell viability. Similar experiments with *sec14^{YL}Δ1* yielded the same results. Finally, we attempted direct substitution of *sec14^{YL}* by *sec14^{YL}Δ2::LEU2^{YL}*, an 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 pINA930 (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^{YL}* is not essential for vegetative growth of *Y. lipolytica*.

To determine if *SEC14^{YL}* is required for spore germination, one of the *SEC14^{YL}* alleles of diploid strain MCL8 (Table I) was replaced by the *sec14^{YL}::URA3^{YL}* 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

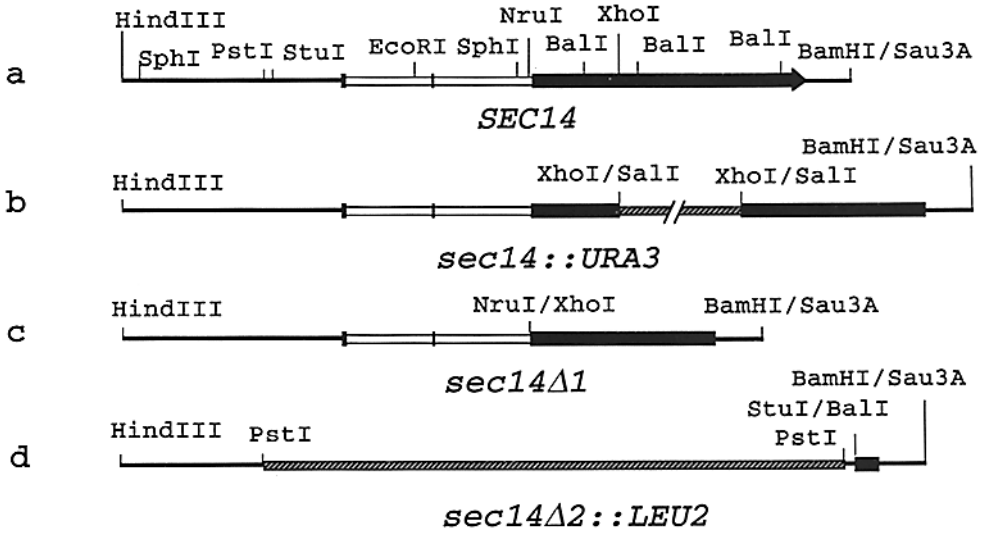

Figure 2. Physical maps of wild-type and mutant *SEC14^{YL}* alleles. (*a*) Wild-type *SEC14^{YL}* cloned as a 3.9-kb HindIII-Sau3a fragment in pINA651, (*b*) *sec14^{YL}::URA3^{YL}* disruption allele in pINA652, (*c*) *sec14Δ1* allele in pINA657, and (*d*) *sec14Δ2::LEU2^{YL}* allele in pINA930. Large 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^{YL}*, *LEU2^{YL}* in *b*, and *d*, respectively.

sec14^YL::URA3^YL as judged by their Ura+ phenotypes. Similar frequencies of inheritance were recorded for the control markers *LYS5^YL* (114/200) and *HIS1^YL* (105/200) which were also segregating in this cross. All Ura+ spores tested carried *sec14^YL::URA3^YL* 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 *sec14^YL::URA3^YL* segregants was typically delayed for approximately one day, relative to *SEC14^YL* progeny, regardless of whether germination occurred on minimal or YPD media (not shown). Nevertheless, these data clearly demonstrate that *SEC14^YL* is not an essential gene in *Y. lipolytica*.

SEC14p^YL Represents the Major PI/PC-TP of Y. lipolytica

We considered several possibilities for why SEC14p^YL is nonessential for *Y. lipolytica* viability. These included that *SEC14^YL* is a duplicated gene, and that SEC14p^YL 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 *SEC14^YL* homologs at the nucleotide sequence level, we generated a radiolabeled probe by PCR using oligonucleotides b and c as synthetic primers and *SEC14^SC* carried on pRE510 as template (Materials and Methods; Table I), and performed hybridizations to the appropriately digested and immobilized genomic DNAs. As demonstrated on Fig. 3*a*, the probe hybridized to the diagnostic 3.7-kb PstI fragment of *SEC14^YL* in wild-type *Y. lipolytica* DNA, and to the expected 3.3-kb PstI fragment of *sec14Δ1* DNA. These experiments were repeated under various stringencies of hybridization with the full-length genomic *SEC14^YL* 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 *SEC14^YL*, these data identify *SEC14^YL* as a unique gene that represents *Y. lipolytica* closest homolog to *SEC14^SC*.

Since the primary sequence of the first 300 SEC14p^YL residues share a 65% identity to that of the entire SEC14p^SC (see above), we used immunoblotting to visualize *Y. lipolytica* polypeptides that are recognized by a polyclonal rabbit anti-SEC14p^SC serum (Bankaitis et al., 1989). These antibodies identified a 58-kD SEC14p^SC-immunoreactive polypeptide in lysates prepared from a wild-type *Y. lipolytica* strain (Fig. 3 *b*) in agreement with predictions derived from *SEC14p^YL* primary sequence. This 58-kD polypeptide species was not detected in lysates prepared from haploid *Y. lipolytica* strains harboring either the *sec14^YL::URA3^YL* or *sec14^YLΔ1* alleles (Fig. 3 *b*).

To determine if SEC14p^YL 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 *sec14Δ1 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 *sec14^YLΔ1* mutant exhibited no significant PI- or PC-transfer activity (Fig. 4). On the basis of our experience with

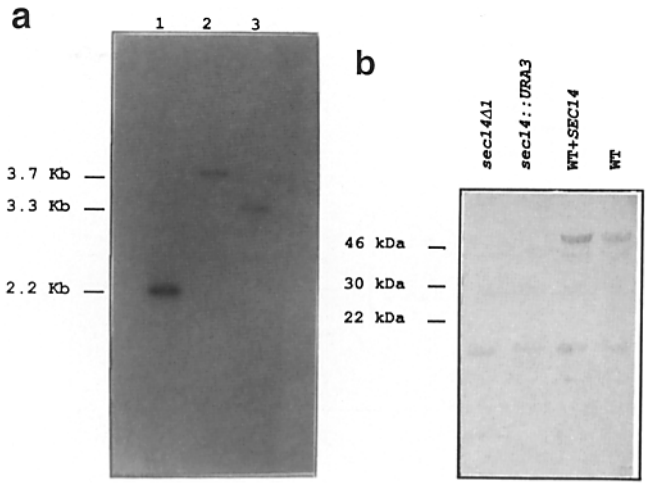

Figure 3. Confirmation of disruption of the *SEC14^YL* locus. (*a*) Southern blot analysis of SEC14-related sequences in *S. cerevisiae* and *Y. lipolytica*. Genomic DNA prepared from *S. cerevisiae* was digested with BamHI and fractionated by agarose (0.7%) gel electrophoresis in lane 1. PstI-cleaved genomic DNA prepared from *Y. lipolytica* strains E122 (wild-type; lane *2*) and MCL12 (*sec14Δ1*; lane *3*) was similarly fractionated. After transfer to Hybond N membrane filters (Amersham, Les Ulis, France) using a Vacugene transfer device (BRL), filters were probed with the 219-bp *SEC14^SC* fragment generated by PCR amplification (see Materials and Methods). Prehybridization and hybridization reactions were at 37°C in 30% formamide, 5× SSC (20× SSC is 175.3 g NaCl and 88.3 g sodium citrate per liter, pH 7.0), 5× Denhardt's solution (100× Denhardt's is 2% Ficoll 400,000, 2% BSA, and 2% polyvinylpyrollidine), 50 mM sodium phosphate, pH 6.0, 150 μg/ml denatured and sonicated herring sperm DNA, and 0.03% SDS. After overnight hybridization, filters were washed 4× for 15-min each in 2× SSC, 0.1% SDS, followed by two washes at 42°C in 0.1× SSC, 0.1% SDS. Size standards are indicated at left. (*b*) Western blot analysis of SEC14p-immunoreactive polypeptides in lysates prepared from the wild-type strain E122 (*WT*), MCL9 (WT+*SEC14*), MCL25 (*sec14^YL::URA3^YL*), and MCL12 (*sec14^YLΔ1*). Strains were grown in minimal YNB medium at 28°C. Cell extracts were prepared, equal amounts of protein were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with a polyclonal rabbit anti-SEC14p^SC serum (see Materials and Methods). Size standards are indicated on the left.

the PI/PC-transfer assays, we estimate that other PL-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 SEC14p^SC and SEC14p^YL extended to a conservation of PI/PC-TP activity, and identified SEC14p^YL 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 SEC14p^YL function for the viability of *Y. lipolytica* is in stark contrast to the essential requirement of SEC14p^SC for the viability of *S. cerevisiae*. How-

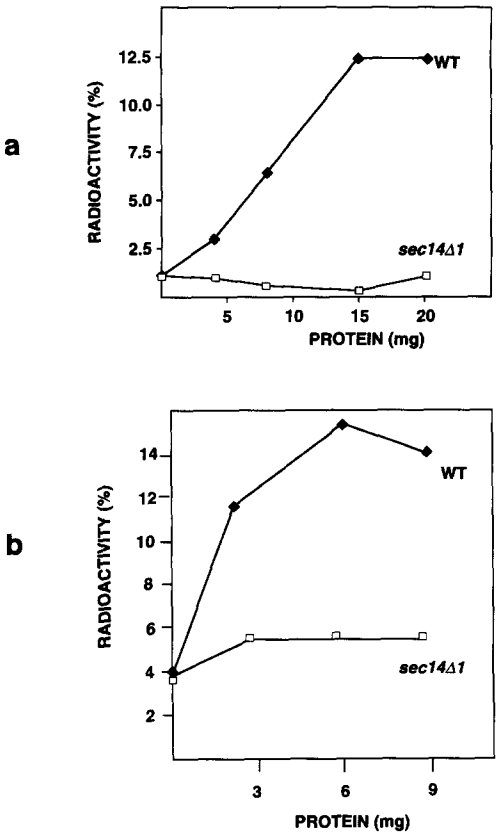

Figure 4. Phospholipid transfer activity in lysates from wild-type and *sec14^{YL}*Δ strains of *Y. lipolytica*. PC (*a*) and PI (*b*) transfer activity was measured in cytosol prepared from the wild-type strain E122 (WT, ♦), and from the *sec14^{YL}Δ1* strain MCL12 (□). Cytosol was incubated with liposomes containing either [^{3}H]-PC or [^{3}H]-PC (transferable lipids), and [^{14}C]cholesteroyl oleate (non-transferable lipid), with purified maize mitochondria (2 mg protein) for 30 min at 30°C. Mitochondria were subsequently resolved 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.

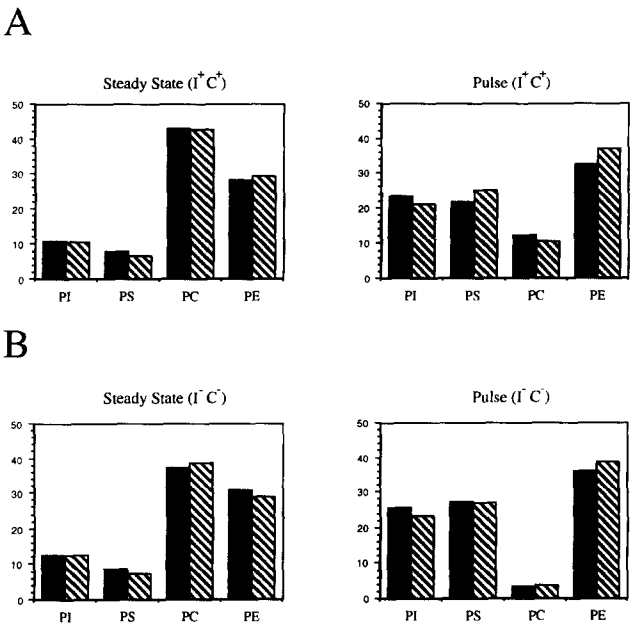

Figure 5. Phospholipid profiles of wild-type and *sec14^{YL} Δ2::LEU2^{YL}* strains of *Y. lipolytica*. The appropriate strains were either grown in medium replete with inositol and choline (*I$^+$C$^+$*) or medium without inositol and choline (*I$^-$C$^-$*), and radiolabeled with [^{32}P]orthophosphate 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 phosphatidylserine (*PS*), phosphatidylethanolamine (*PE*), phosphatidylcholine (*PC*), and phosphatidylinositol (*PI*) 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 (PO1a) are represented by the solid bars, whereas values for the *sec14^{YL}Δ2::LEU2^{YL}* 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.

ever, one mechanism for alleviating the essential SEC14p^{SC} requirement of *S. cerevisiae* involves the inactivation of the CDP-choline pathway for PC biosynthesis via the CDP-choline pathway (Kennedy and Weiss, 1956; Cleves et al., 1991*b*). To test whether the nonessentiality of SEC14p^{YL} might reflect a natural incompetence of *Y. lipolytica* for PC synthesis via the CDP-choline pathway, we estimated bulk PL biosynthetic rates and measured bulk membrane steady state PL compositions of wild-type and *sec14^{YL}Δ2::LEU2^{YL}* strains of *Y. lipolytica* cultured either in medium supplemented with both inositol and choline (I$^+$C$^+$), or in medium lacking both of these nutrients (I$^-$C$^-$) (Fig. 5).

When either wild-type or *sec14^{YL}Δ2::LEU2^{YL}* strains of *Y. lipolytica* were cultured in I$^-$C$^-$ medium and subjected to a 30 min pulse-radiolabeling with $^{32}P_i$, both strains exhibited similar radiolabeled bulk membrane PL profiles that consisted of approximately 25 mole% PI, 27 mole% phosphatidylserine (PS), 3 mole% PC, and 37 mole% phosphatidylethanol-amine (PE). These data suggested that wild-type and *sec14^{YL}Δ2::LEU2^{YL}* strains exhibited similar rates of synthesis for each of these four PL species. The steady state bulk membrane compositions of these strains grown in I$^-$C$^-$ 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 *sec14^{YL}Δ2::LEU2^{YL}* in I$^+$C$^+$ or I$^-$C$^-$ medium did not alter the steady state bulk membrane profiles of these strains, a significant change in the apparent rate of PC biosynthesis was recorded in I$^+$C$^+$ grown cells relative to I$^-$C$^-$ grown cells. Specifically, both wild-type and *sec14^{YL}Δ2::LEU2^{YL}* strains exhibited approximately 12 mole% PC in the pulse-radiolabeled bulk PL profile (Fig. 5 *B*). This stimulation represented an almost four-fold increase in the apparent rate of PC synthesis in these strains when cultured in I$^+$C$^+$ medium, as opposed to I$^-$C$^-$ medium, and depended on the presence of choline and not the presence of inositol (not shown). As PC produced under conditions of pulse-radiolabeling predominantly reflects activity of the CDP-choline pathway (McGee et al., 1994), this choline-dependent stimulation of PC synthesis revealed a functional CDP-choline pathway in *Y. lipolytica*. This con-

clusion was directly confirmed by demonstrating the incorporation of [^{14}C]choline into *Y. lipolytica* bulk membrane PC, and the presence of CDP–choline pathway intermediates (i.e., phosphorylcholine and CDP–choline) in *Y. lipolytica* cells (not shown). These collective data demonstrate that SEC14p^{YL} is not involved in controlling bulk membrane composition in *Y. lipolytica*, and that the nonessentiality of SEC14p^{YL} is not the result of a natural CDP–choline pathway deficiency in this yeast. We also note that bulk *Y. lipolytica* 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^{YL} 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 morphology on plates (Rodriguez and Dominguez, 1984). Thus, mutants 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 *SEC14^{YL}* allele had been replaced by *sec14^{YL}::URA3^{YL}*, *sec14^{YL}Δ1*, or *sec14^{YL}Δ2::LEU2^{YL}* 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 *sec14^{YL}::URA3^{YL}* (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 *sec14^{YL}::URA3^{YL}* 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 *sec14^{YL}::URA3^{YL}* strain was grown on media favoring hyphae formation (not shown). This strain did, however, acquire a pseudomycelial morphology upon entry into stationary phase with a concomitant formation of cell aggregates (Fig. 6 *b*).

To further confirm that the morphological defect observed for *sec14^{YL}* strains was the result of SEC14p^{YL} dysfunction, we tested whether reintroduction of *SEC14^{YL}* into such strains could reverse the morphological defect. An autonomously replicating *LEU2^{YL}*, *SEC14^{YL}* plasmid (pINA653; see Table I) was transformed into a *sec14^{YL}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^{YL} dysfunction.

Subcellular Localization of SEC14p^{YL}

SEC14p^{SC} localizes as a peripheral membrane protein of the *S. cerevisiae* Golgi complex (Cleves et al., 1991*b*). This assignment 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., 1991*b*). KEX2p is a protease that is involved in the proteolytic processing of specific prohormone precursors at sites of dibasic residues (Fuller et al., 1989). Since the phenotypic 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^{YL}.

Treatment of fixed wild-type *Y. lipolytica* cells with a primary 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^{YL} specific since: (*a*) exclusion of the anti-SEC14p^{SC} antibodies from the staining regimen; or (*b*) use of fixed *sec14^{YL}Δ2::LEU2^{YL}* cells in the complete staining regimen, led to a complete loss of FITC staining (not shown). Moreover, these SEC14p^{YL} positive structures failed to colocalize with mitochondria or the nucleus as judged by comparing the FITC and DAPI staining profiles. We did note, however, that SEC14p^{YL} positive structures frequently exhibited some clustering in a perinuclear 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^{YL} positive structures.

To determine whether SEC14p^{YL} is associated with *Y. lipolytica* Golgi bodies, we attempted to identify *Y. lipolytica* Golgi bodies, using a primary mouse anti-KEX2p antiserum and appropriate Texas red–conjugated antibodies (see Materials and Methods). The rationale for using these primary 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* encodes an endoprotease that has been implicated in KEX2p-like proteolytic processing, at dibasic residues, of the alkaline 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). Finally, immunoblotting of *Y. lipolytica* lysates with anti-KEX2p serum reveals a single KEX2p-immunoreactive species (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^{YL}, 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^{YL}, 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 detection of Texas red signal (not shown).

Superimposition of the SEC14p^{YL} and XPR6p profiles by digital image processing revealed a high degree of colocal-

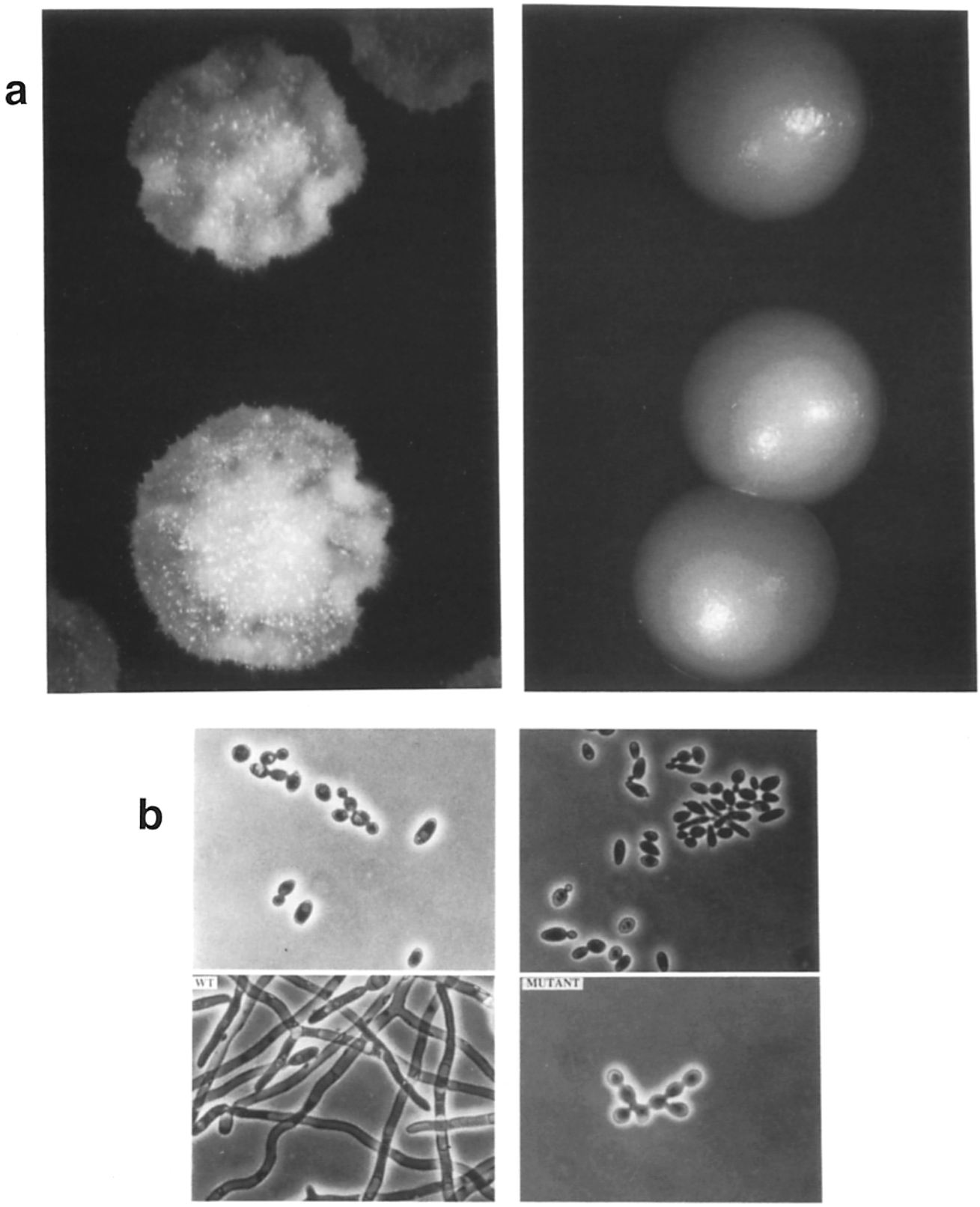

Figure 6. *secl4*^{YL} strains fail to execute the transition from yeast to mycelium. Wild-type (MCL28) and *secl4*^{YL}*::URA3*^{YL} (MCL30) strains were grown on either solid (*a*) or liquid (*b*) YPD medium at 28°C. (*a*) Colony morphology after 36 h of growth on solid YPD medium is typified by the rough phenotype for the wild-type strain (*left*) and the smooth phenotype for the *secl4*^{YL}*::URA3*^{YL} 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 *secl4*^{YL}*::URA3*^{YL} strain (MCL30, *right*).

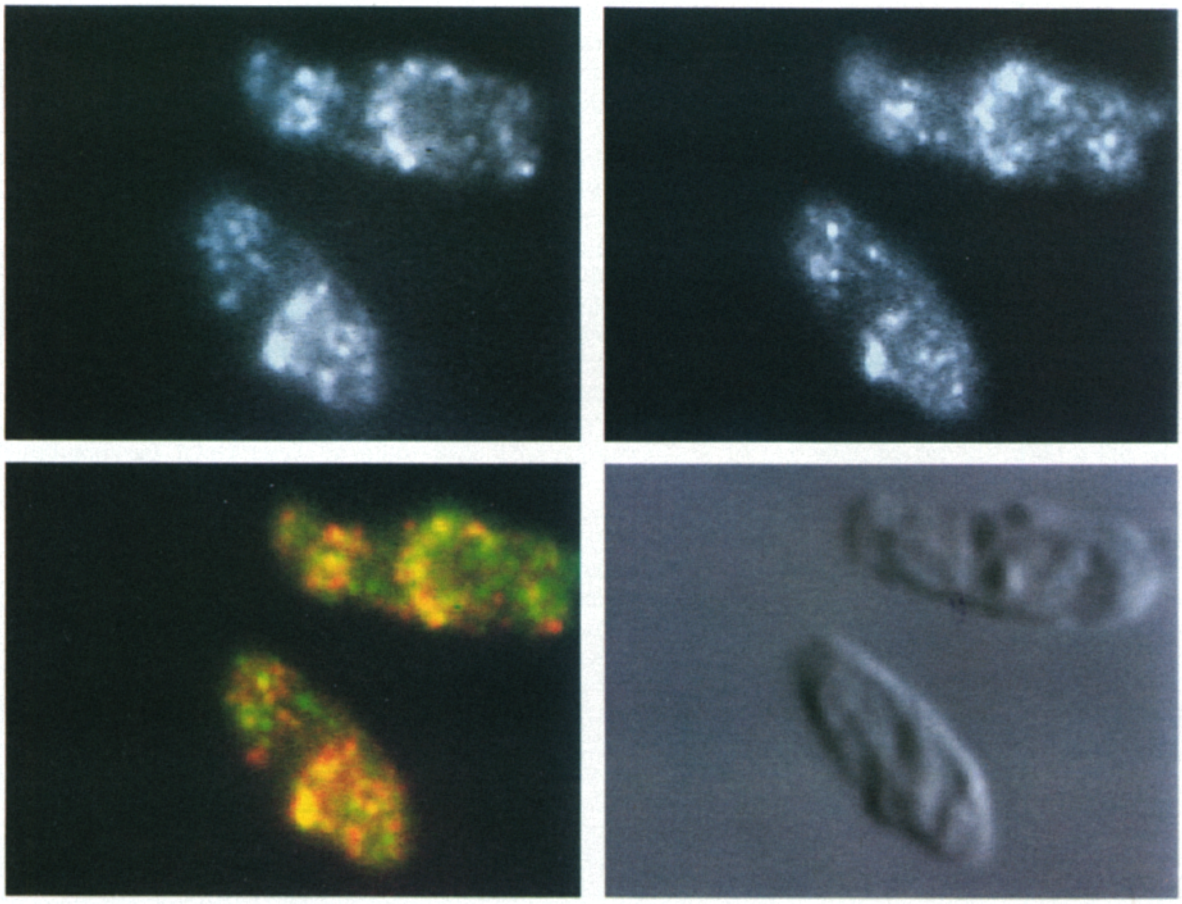

Figure 7. SEC14p^{YL} colocalizes with the presumptive Golgi marker XPR6p. The wild-type *Y. lipolytica* strain POla was grown to early logarithmic phase in YNB medium, cells were fixed in 3.7% formaldehyde, and prepared for immunofluorescence microscopy as described in Materials and Methods. The XPR6p profile was revealed by sequential incubation of cells with a primary mouse polyclonal antibody raised against the *S. cerevisiae* KEX2p, a secondary polyclonal sheep anti-mouse antiserum, and Texas red-conjugated donkey anti-sheep antibodies (*top left*). The SEC14p^{YL} profile was revealed by serial incubation of these same cells with a primary rabbit antiserum directed against the *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^{YL} signals were completely abolished by either the exclusion of the corresponding primary antibodies from the staining regimen, or by introduction of Δ*xpr6* and Δ*sec14*^{YL} alleles into the *Y. lipolytica* strain to be tested.

ization between these two markers (Fig. 7). Points of complete coincidence between XPR6p and SEC14p^{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^{YL} stained positive for both. Extensive coincidence of staining was especially apparent in the perinuclear region. These data demonstrate that XPR6p and SEC14p^{YL} exhibit a high, but not absolute, degree of colocalization to a disperse cytoplasmic organelle of the *Y. lipolytica* secretory pathway, likely the Golgi complex.

sec14^{YL} *Mutants Exhibit Wild-type Secretory Pathway Function*

The viability of Δ*sec14*^{YL} strains indicated that SEC14p^{YL} was largely dispensable for secretory functions in *Y. lipolytica*, the localization of SEC14p^{YL} to what are presumed to be *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 *sec14*^{YL} mutant stains. The *sec14*^{YL}*::URA3*^{YL} allele interrupts SEC14p^{YL} after residue 163 (Fig. 1 and 2). As interruption of SEC14p^{SC} at residue 234 represents a null mutation (i.e., *sec14Δ1::HIS3*; Bankaitis et al., 1989), we believe that *sec14*^{YL}*::URA3*^{YL} 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 *sec14*^{YL}*::URA3*^{YL} 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 *sec14*^{YL}*::URA3*^{YL} 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 *sec14*^{YL}*::URA3*^{YL} 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 *sec14^{YL}::URA3^{YL}* 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. 8 *C*). 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 *sec14^{YL}::URA3^{YL}* strain. These data indicated that AEP transit through the early stages of the *Y. lipolytica* secretory pathway was not affected in *sec14^{YL}::URA3^{YL}* 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. 8 *C*. In both wild-type and *sec14^{YL}::URA3^{YL}* 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 SEC14p^{YL} dysfunction.

Discussion

The study of SEC14p^{SC} 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 SEC14p^{SC} for Golgi secretory function and cell viability (Cleves et al., 1989; Cleves et al., 1991*b*). 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 SEC14p^{SC} 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 SEC14p^{SC} 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., 1991*a*,*b*). However, while subsequent biochemical analyses indicate that SEC14p^{SC} 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 SEC14p^{SC} 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 SEC14p^{SC} is believed to somehow reflect an essential functional property of SEC14p^{SC} as evidenced by the ability of a mammalian PI/PC-TP, which exhibits no primary se-

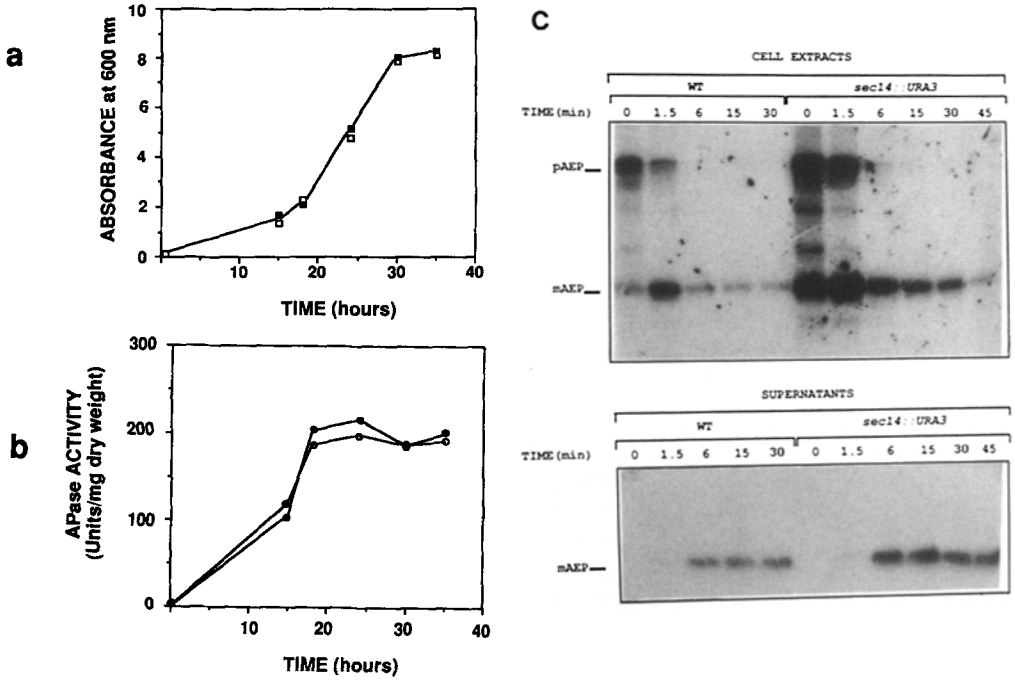

Figure 8. Protein secretion in wild-type and *sec14^{YL}* strains. Isogenic *Y. lipolytica* strains were monitored for their growth (*a*), periplasmic phosphatase activity (*b*), and secretion of alkaline extracellular protease (*c*). The two strains, MCL28 (*SEC14^{YL}*) and MCL30 (*sec14^{YL}::URA3^{YL}*), 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 OD_{600} (*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 [^{3}H] leucine (3,000 Ci/mmole, L-[4,5-^{3}H]-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.

quence similarity to SEC14p^{SC}, to complement the *S. cerevisiae sec14-1^{ts}* mutation (for a discussion see Skinner et al., 1993). Moreover, although it is not yet precisely clear as to why SEC14p^{SC}-mediated control of Golgi membrane PC content is an essential activity in *S. cerevisiae*, it has recently been suggested that SEC14p^{SC} 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 SEC14p^{SC} as a paradigm for the in vivo function of a PL-TP is how generally applicable are the basic features of the SEC14p^{SC} 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 SEC14p^{SC}, coupled with the ability of expression of a mammalian PI/PC-TP to correct *sec14-1^{ts}* 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*a*; Skinner et al., 1993). A rigorous test of this possibility requires the availability of *sec14* mutants in other organisms. For this reason, we characterized SEC14p^{YL} 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* (Barns et al., 1991); and (*c*) for its unusual capacity to secrete high levels of protein into the extracellular medium.

Our data indicate that, while certain aspects of SEC14p^{YL} function are conserved with respect to those of SEC14p^{SC}, these polypeptides control different biological processes in their respective host organisms. The similarities are severalfold. First, the SEC14p^{YL} is a 497-amino acid polypeptide whose first 300 residues exhibit a 65% identity to the 304-amino acid SEC14p^{SC}, and a 66% identity to the 301 residue SEC14p^{KL} (Fig. 1). This level of primary sequence conservation is commonly observed when *Y. lipolytica* gene products are compared to their *S. cerevisiae* counterparts (Davidow et al., 1987; Strick et al., 1992). Although SEC14p^{YL} exhibits a 197-residue carboxy-terminal extension that is not represented in SEC14p^{SC}, SEC14p^{KL}, or SEC14p^{SP}, we have thus far failed to detect an important role for this domain in SEC14p^{YL} function in vivo as all consequences associated with loss of SEC14p^{YL} function in *Y. lipolytica* (e.g., loss of measurable intracellular PI/PC-TP activity and impairment of the developmental switch from the yeast to the mycelial mode of growth) are corrected by expression of SEC14p^{YL} that lacks this COOH-terminal domain. The only detectable consequence of deletion of this domain is an apparent reduction in SEC14p^{YL} stability in vivo (not shown). Thus, the SEC14p^{SC}-like domain of SEC14p^{YL} represents its functional domain. Second, the ability of SEC14p^{YL} expression to complement the lethality of *sec14* null mutations in *S. cerevisiae* indicates that SEC14p^{YL} fulfills all of the essential SEC14p^{SC} functions in the heterologous system. The biochemical basis for the functional relatedness between SEC14p^{SC} and SEC14p^{YL} was confirmed by: (*a*) in vitro experiments that demonstrated SEC14p^{YL} to be a PI/PC-TP; and (*b*) that, as expected, it was the functional SEC14p^{SC}-like domain of SEC14p^{YL} that represented its PI/PC-TP domain (Fig. 4). Finally, double-label immunofluorescence experiments demonstrated a substantial, albeit not absolute, colocalization of SEC14p^{YL} with the presumptive *Yarrowia* Golgi marker XPR6p (Fig. 7). The general pattern of SEC14p^{YL} and XPR6p staining was a disperse punctate staining in the *Yarrowia* cytoplasm, much like that which has been observed for Golgi-specific staining in *S. cerevisiae* and *S. pombe* (Franzusoff et al., 1991; Cleves et al., 1991*b*; 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 SEC14p^{SC} in *S. cerevisiae*, SEC14p^{YL} 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 SEC14p^{YL} with compartments of the *Yarrowia* secretory pathway is probable.

A surprising distinction between SEC14p^{SC} and SEC14p^{YL} function in vivo was forthcoming from gene disruption experiments. Whereas SEC14p^{SC} is essential for the viability of wild-type *S. cerevisiae* (Bankaitis et al., 1989), SEC14p^{YL} clearly was not required for the viability of *Y. lipolytica* under conditions of vegetative growth or germination from spores. Moreover, SEC14p^{YL} 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^{YL}* strains (Fig. 8, *b* and *c*). The nonessentiality of SEC14p^{YL} is not readily explained by the presence of some redundant function as evidenced by our inability to detect SEC14p^{YL} homologs by nucleic acid hybridization experiments or immunoblotting experiments with anti-SEC14p^{SC} serum (Fig. 3, *a* and *b*). Moreover, phospholipid transfer assays using lysates prepared from wild-type and *sec14^{YL}* mutant strains of *Y. lipolytica* identified SEC14p^{YL} as the major, and perhaps the only, *Yarrowia* PI/PC-TP (Fig. 4). Thus, the available evidence leads us to conclude that SEC14p^{YL} (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 SEC14p^{YL} could result from *Yarrowia* being naturally incompetent for PC synthesis via this pathway (Fig. 5); a mechanism by which the normally essential SEC14p^{SC} function can be bypassed in *S. cerevisiae* (Cleves et al., 1991*b*). These data further reinforce the distinction between the in vivo functions of SEC14p^{SC} and SEC14p^{YL}. Thus, irrespective of the significant aspects of functional similarity shared by SEC14p^{SC} and SEC14p^{YL}, these PI/PC-TPs are involved in controlling distinct physiological processes in their respective host organisms. Finally, the finding that SEC14p^{YL} 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

membrane lipid from the late stages of the secretory pathway back to the ER (Wieland et al., 1987; Rothman, 1990; Cleves et al., 1991*a*,*b*).

While SEC14p^{YL} function is not essential for *Yarrowia* viability, our genetic data demonstrated a requirement for SEC14p^{YL} in the dimorphic transition of the yeast form into the mycelial form. *Yarrowia sec14^{YL}* 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 (ovoid to filamentous), mode of culture growth (exponential to linear), and mechanism of cell division (budding mode to septation). The inability of *sec14^{YL}* strains to assume a true mycelial form when the culture achieves stationary phase (Fig. 6 *b*), indicates a role for SEC14p^{YL} in promoting at least one of these transition steps.

How might SEC14p^{YL} 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^{YL} and SEC14p^{SC} may play mechanistically divergent roles in their respective organisms. However, there is in vivo evidence to suggest that, although the primary function of SEC14p^{SC} appears to be a regulatory activity directed at the control of Golgi membrane PC content (McGee et al., 1994), the ultimate consequence of SEC14p^{SC} 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., 1991*a*; 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 sacl mutants that exhibit: (*a*) the ability to suppress mutations in the actin structural gene of yeast in an allele-specific manner (Novick et al., 1989); (*b*) the ability to suppress *sec14^{SC}* 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^{SC} and SEC14p^{YL} 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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