

# A Phosphatidylinositol Transfer Protein Controls the Phosphatidylcholine Content of Yeast Golgi Membranes

Todd P. McGee, Henry B. Skinner, Eric A. Whitters, Susan A. Henry,\* and Vytas A. Bankaitis

Department of Cell Biology, University of Alabama at Birmingham, Birmingham, Alabama 35294-0005; and \* Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213

**Abstract.** SEC14p is required for protein transport from the yeast Golgi complex. We describe a quantitative analysis of yeast bulk membrane and Golgi membrane phospholipid composition under conditions where Golgi secretory function has been uncoupled from its usual SEC14p requirement. The data demonstrate that SEC14p specifically functions to maintain a reduced phosphatidylcholine content in Golgi membranes and indicate that overproduction of SEC14p

markedly reduces the apparent rate of phosphatidylcholine biosynthesis via the CDP-choline pathway *in vivo*. We suggest that SEC14p serves as a sensor of Golgi membrane phospholipid composition through which the activity of the CDP-choline pathway in Golgi membranes is regulated such that a phosphatidylcholine content that is compatible with the essential secretory function of these membranes is maintained.

**T**HE *Saccharomyces cerevisiae* SEC14 gene product (SEC14p) is a phosphatidylinositol/phosphatidylcholine transfer protein whose function is essential for both protein transport from a late yeast Golgi compartment and for yeast viability (Bankaitis et al., 1989, 1990; Franzusoff and Schekman, 1989; Aitken et al., 1990). As such, SEC14p has provided a system with which to study the *in vivo* function of a ubiquitous class of enigmatic proteins, the phospholipid transfer proteins, that are operationally defined by their ability to serve as diffusible carriers of phospholipid monomers between membrane bilayers *in vitro* (reviewed by Rueckert and Schmidt, 1990; Wirtz, 1991; Cleves et al., 1991a). We consider SEC14p to play a direct role in yeast Golgi secretory function as this protein is found in both a cytoplasmic pool and in a stable, and apparently specific, peripheral association with the yeast Golgi complex (Bankaitis et al., 1989; Cleves et al., 1991b).

To date, the most instructive clues relating to SEC14p function *in vivo* have been obtained from analyses of yeast mutants that no longer require SEC14p in order to survive and efficiently execute Golgi secretory function (Cleves et al., 1989, 1991b). These studies revealed that one unanticipated mechanism for bypass of SEC14p function involves inactivation of the cytosine-diphosphate (CDP)-choline pathway for phosphatidylcholine (PC)<sup>1</sup> biosynthesis, a pathway that

consists of three reactions resulting in the incorporation of choline into PC (Kennedy and Weiss, 1956; see Fig. 1 A). The finding that the cellular requirement for SEC14p is obviated by inactivation of a specific avenue for PC biosynthesis demonstrated, for the first time, a direct physiological relationship between an *in vitro* ligand of a phospholipid transfer protein and the function of that phospholipid transfer protein *in vivo*. The collective SEC14p data, including the data indicating that SEC14p prefers phosphatidylinositol (PI) over PC as a substrate in the *in vitro* transfer reaction (Daum and Paltauf, 1984), have been reconciled in the PI/PC hypothesis for SEC14p function *in vivo* (Cleves et al., 1991a,b). The PI/PC hypothesis proposes that SEC14p functions to maintain an appropriately elevated PI/PC ratio in Golgi membranes, a ratio that is somehow critical to the secretory competence of these membranes (Fig. 1 B). The central questions to be addressed include: (a) what is the functional basis for the unanticipated relationship between PC synthesis via the CDP-choline pathway and the cellular requirement for SEC14p function; (b) does SEC14p in fact regulate the PI/PC ratio of yeast Golgi membranes *in vivo*; and (c) does this control involve SEC14p-dependent changes in Golgi PI levels, Golgi PC levels, or both (Bankaitis et al., 1990; Cleves et al., 1991a). Moreover, any comprehensive model for SEC14p function *in vivo* must also take into account three puzzling features of the suppression of SEC14p dysfunction by inactivation of the CDP-choline pathway (reviewed in Cleves et al., 1991b; McGee et al., 1992). The first concerns the finding that, while elimination of the contribution of the CDP-choline pathway to net PC biosynthesis in yeast (i.e., by depriving cells of exogenous choline) fails to bypass the cellular requirement for SEC14p, disruption of the CDP-choline pathway nonetheless effects bypass of

Address all correspondence to Dr. V.A. Bankaitis, Department of Cell Biology, 668 Basic Health Sciences Building, University of Alabama at Birmingham, Birmingham, AL 35294-0005.

**1. Abbreviations used in this paper:** BCA, bichononic acid; BHT, butylated hydroxytoluene; CCTase, cholinephosphate cytidyltransferase; CDP, cytosine-diphosphate; GMP, Golgi Integral Membrane Proteins; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

SEC14p in choline-free medium. The second relates to the question of why disruption of the *sn*-1,2-diacylglycerol cholinephosphotransferase structural gene (*CPT1*) effects a bypass of the SEC14p requirement whereas disruption of the *sn*-1,2-diacylglycerol ethanolamine-phosphotransferase structural gene (*EPT1*) does not (Cleves et al., 1991b). These enzymes are homologous aminoalcohol-phosphotransferases and both can efficiently catalyze the ultimate PC biosynthetic reaction of the CDP-choline pathway in vitro (Hjelmstad and Bell, 1987, 1988). The third feature is the finding that genetic disruption of the PE methylation pathway for PC biosynthesis (see Fig. 1 A) is irrelevant to the cellular requirement for SEC14p.

In this report, we describe the measurement of yeast Golgi membrane phospholipid composition as a function of SEC14p activity. We demonstrate that, under conditions where Golgi secretory function has been uncoupled from the SEC14p requirement, SEC14p dysfunction results in a dramatic increase in the PC content of Golgi membranes without measurable changes in Golgi PI levels. Double-mutant analyses indicate that the increase in Golgi PC measured under such conditions is accounted for by CDP-choline pathway activity. We also demonstrate that CDP-choline pathway function is required for the maintenance of normal phosphatidylcholine biosynthetic rates in bulk yeast cell membranes, and that this requirement is independent of the presence or absence of choline in the growth medium. Finally, we present evidence that overproduction of SEC14p results in a specific reduction in bulk PC biosynthesis via the CDP-choline pathway, but not via the PE methylation pathway. On the basis of these collective data, we conclude that the essential cellular function of SEC14p is to maintain a reduced PC content in yeast Golgi membranes and suggest an alternative proposal for how SEC14p executes this in vivo function in a manner that does not invoke phospholipid transfer per se. We suggest the possibility that SEC14p may serve as a dynamic sensor of the relative PI and PC content in Golgi membranes through which the local activity of the CDP-choline biosynthetic pathway in yeast Golgi membranes is regulated, so that an acceptable PC content can be maintained.

## Materials and Methods

### Yeast Strains, Media, and Reagents

The genotypes of the yeast strains used in this study are provided in Table I. Yeast complex medium (YP), YP supplemented with glucose to a final concentration of 2% (YPD), and synthetic complete media have been previously described by Sherman et al. (1983) and Klig et al. (1985), respectively. *myo*-Inositol and choline chloride were purchased from Sigma Immunochemicals (St. Louis, MO) and were used to supplement synthetic defined media to final concentrations of 1 mM each as indicated. [<sup>32</sup>P]Orthophosphate (carrier-free) and [<sup>14</sup>C]choline chloride (40–60 mCi/mmol) were obtained from New England Nuclear (Boston, MA). [<sup>14</sup>C]Choline chloride (55 mCi/mmol) and L-[*methyl*-<sup>14</sup>C]methionine (55 mCi/mmol) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Silica-impregnated SG81 chromatography paper and rhodamine 6G were from Whatman Laboratory Products (Clifton, NJ) and Sigma Immunochemicals, respectively. The remaining reagents and routine genetic techniques used were described by Cleves et al. (1991b).

### Determination of Bulk Phospholipid Content by Radiolabeling with [<sup>32</sup>P]Orthophosphate

Pulse labeling of the appropriate yeast strains was performed on cultures grown to mid-logarithmic growth phase (OD<sub>600</sub> = 0.8) in the synthetic complete medium of Klig et al. (1985) and incubated with [<sup>32</sup>P]orthophosphate (<sup>32</sup>P<sub>i</sub>; 10 μCi/ml) for 30 min at 25°C with shaking. For steady-state <sup>32</sup>P<sub>i</sub>-labeling experiments, yeast were grown in synthetic complete medium overnight then subcultured into the same fresh medium. The cultures were then presented with <sup>32</sup>P<sub>i</sub> (10 μCi/ml) and incubated at 25°C with shaking for five to six cell generations, a sufficiently long period of incorporation of <sup>32</sup>P<sub>i</sub> to insure 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) in a clinical centrifuge, washed by incubation in ice cold TCA (5%) for 20 min with subsequent repelleting, and the cell pellet was resuspended in 1 ml polar extraction solvent (Steiner and Lester, 1972) with heating at 65°C for 20 min. Phospholipids were recovered from the cell suspension by the addition of 5.0 ml CHCl<sub>3</sub>/CH<sub>3</sub>OH/butylated hydroxytoluene (BHT) (2:1:0.0005%), followed by incubation at 4°C for 2–6 h. The extraction cocktails were subsequently centrifuged at 500 g for 5 min to separate the organic and aqueous phases. The organic phase was removed, dried under N<sub>2</sub> gas, and resuspended in 60 μl CHCl<sub>3</sub>/CH<sub>3</sub>OH/BHT. Radiolabeled phospholipids were subsequently resolved by two-dimensional chromatography using Whatman SG81 paper treated as described by Steiner and Lester (1972). First dimension solvent was CHCl<sub>3</sub>/CH<sub>3</sub>OH/NH<sub>4</sub>OH/H<sub>2</sub>O (22:9:1:0.26) and second dimension solvent was CHCl<sub>3</sub>/CH<sub>3</sub>OH/CH<sub>3</sub>COOH/H<sub>2</sub>O (8:1:1.25:0.25). Finally, radiolabeled phospholipids were visualized by autoradiography, identified by comparison to commercial phospholipid standards, and defined phospholipid species were recovered by excision from the chromatography paper. Quantitation of individual phospholipid species was achieved by scintillation counting.

In experiments where phospholipid composition was determined as a function of viable cell number, parallel cultures were initiated from the same starter culture and were co-incubated. One culture received radiolabel for the appropriate period of time and was reserved for phospholipid analysis, whereas the other culture did not receive radiolabel and was reserved for determination of viable cell number. The culture reserved for viable cell counting was quick-chilled in an ice-water bath, appropriately diluted, and stained with 4 mg/ml Phloxine B to distinguish between viable (unstained) and nonviable (red-staining) cells. Viable cells were counted with the aid of a hemacytometer (Hausser Scientific, Boulder, CO). Budding cells in which the daughter cells were judged to be less than half the size of the parental cell were recorded as single cells, whereas budding cells in which the buds were judged to exceed half the size of the mother cell were counted as two cells. The timing of the chilling of the culture reserved for viable cell counting coincided with the extraction of phospholipid from the radiolabeled culture. Glycerophospholipids were extracted from the radiolabeled culture and analyzed as described above.

### [<sup>14</sup>C]Choline Labeling of Yeast Lipids

The appropriate yeast strains were grown to mid-logarithmic growth phase in defined medium lacking choline, and presented with [<sup>14</sup>C]choline chloride (1 μCi/ml) for 30 min at 25°C with shaking. Incorporation of label was measured by removing one-tenth of the culture, immobilization of the culture aliquot on 0.5 μm glass fiber filters, washing of the filters 4× with 2.5 vol of 10 mM choline per wash, and resuspension of the dried cell pellets and filters to which these were immobilized in scintillation fluid for counting. [<sup>14</sup>C]Choline incorporation values were used to normalize loading of lipid samples derived from each of these cultures for chromatographic analysis. Phospholipids were extracted from the remainder of each culture, as described above, and normalized amounts of lipid sample from each culture were loaded onto Whatman SG81 paper for resolution by chromatography in one dimension using a solvent system of CHCl<sub>3</sub>/CH<sub>3</sub>OH/NH<sub>4</sub>OH (22:5.7:1). [<sup>14</sup>C]Choline-containing lipids were visualized by autoradiography using Kodak X-OMAT AR film.

In time course studies, the appropriate yeast strains were grown to mid-logarithmic growth phase in defined medium lacking choline, concentrated to 2 OD<sub>600</sub> U/ml, and allowed to incorporate [<sup>14</sup>C]choline chloride (1 μCi/ml) into phospholipid at 25°C in a 1 ml culture volume as a function of time. Incorporation of label into cells was measured by removing one-

Table I. Yeast Strains

Strain	Genotype	Origin
CTY182	<i>MAT a, ura3-52, Δhis3-200, lys2-801</i>	Bankaitis et al. (1989)
CTY1-1A	<i>MAT a, ura3-52, Δhis3-200, lys2-801, sec14-1<sup>ts</sup></i>	Bankaitis et al. (1989)
CTY10	<i>MAT α, ade2-101, sec14-1<sup>ts</sup>, sac1-16<sup>ts</sup></i>	This study
CTY31	<i>MAT α, ade2-101, sec14-1<sup>ts</sup>, bsd2-1</i>	This study
CTY105	<i>MAT a, ura3-52, Δhis3-200, lys2-801, sec14-1<sup>ts</sup>, bsr2-5</i>	This study
CTY124	<i>MAT a, ura3-52, Δhis3-200, lys2-801, sec14-1<sup>ts</sup>, bsd1-124</i>	Cleves et al. (1991)
CTY156	<i>MAT a, ura3-52, Δhis3-200, ade2-101, lys2-801, sec14-1<sup>ts</sup>, bsr3-2</i>	This study
CTY234	<i>MAT a, ura3-52, Δhis3-200, lys2-801/YEp(KEX2)</i>	Whitters et al. (1993)
CTY392	<i>MAT a, ura3-52, Δhis3-200, lys2-801, sec14-1<sup>ts</sup>, cki-284::HIS3</i>	This study
CTY434	<i>MAT a, ura3-52, his4-519, ade2-101, leu2-3, 112, sec14-1<sup>ts</sup>, cpt1::LEU2</i>	This study
CTY479	<i>MAT α, ura3-52, Δhis3-200, ade2-101, bsd2-1</i>	This study
CTY521	<i>MAT α, ura3, Δhis3, lys2, trp1, leu2/YEp(SEC14)</i>	This study
CTY525	<i>MAT α, ura3, Δhis3, lys2, trp1, leu2/YEp(lac112)</i>	This study

tenth of the culture, harvesting the cells in that culture aliquot by rapid filtration onto 0.5- $\mu$ m glass fiber filters, washing of the filters 4 $\times$  with 50 vol of 10 mM choline per wash, and resuspension of the dried filters in scintillation fluid for counting. Phospholipids were extracted, resolved, and visualized from the remainder of each culture, as described above, and [<sup>14</sup>C]choline-labeled phospholipids quantitated by scintillation counting.

### Organelle Marker Assays

Endoplasmic reticulum, mitochondrial membrane, and plasma membrane markers were NADPH cytochrome c reductase, cytochrome c oxidase, and vanadate-sensitive ATPase, respectively, and these enzymatic activities were assayed by established methods (Kreibich et al., 1973; Mason et al., 1973; Bowman and Slayman, 1979). Vacuolar, mitochondrial and Golgi membrane markers were dipeptidylaminopeptidase B, F<sub>1</sub>-ATPase B subunit and KEX2p, respectively, and these markers were quantitated by ELISA as described by Cleves et al. (1991b). Antibody concentrations for these ELISA measurements were 10.2  $\mu$ g/ml, 6.7  $\mu$ g/ml, and 3.4  $\mu$ g/ml, respectively. The dipeptidylaminopeptidase B, F<sub>1</sub>-ATPase B subunit, and KEX2p antibodies were from polyclonal rabbit antisera generously provided by Tom Stevens (University of Oregon, Eugene, OR), David Bedwell (University of Alabama at Birmingham, Birmingham, AL), and Robert Fuller (Stanford University, Stanford, CA), respectively.

### Preparation of Polyclonal Mouse anti-GMP Antibody

Golgi membranes from the most highly enriched Golgi fractions (see below for preparative details) were diluted threefold in 10 mM triethanolamine buffer, pH 7.2, supplemented with protease inhibitors (Cleves et al., 1991b). The membranes were then pelleted onto a 4.3 M sorbitol cushion by centrifugation at 250,000 g for 3 h in an ultracentrifuge (TLS-100; Beckman Instruments, Palo Alto, CA). Pelleted membranes were collected, resuspended in an equal volume of 200 mM Na<sub>2</sub>CO<sub>3</sub>, pH 11.5, and incubated on ice for 60 min. The membranes were subsequently repelleted at 250,000 g as before, re-extracted in 1.5 ml of fresh 100 mM Na<sub>2</sub>CO<sub>3</sub>, pH 11.5, by incubation on ice for 60 min, and repelleted. Finally, the extracted membranes were solubilized in 0.1% Triton X-100 and the integral membrane protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit marketed by Pierce Chemical Co. The solubilized integral membrane proteins were subsequently emulsified in an equal volume of Freund's incomplete adjuvant and injected i.p. into Balb/C mice (125  $\mu$ g/injection). A serial 3-wk injection schedule was followed, and the antibody response was monitored by immunoblot analysis. Serum lipoproteins were cleared from immune serum by precipitation with CaCl<sub>2</sub> and dextran sulfate before use.

### Indirect Immunofluorescence Methods

Crude polyclonal anti-GMP antisera was affinity purified using a GMP-Sepharose affinity column that was prepared using a modification of an established procedure (Skinner et al., 1993). Briefly, Golgi integral membrane proteins (prepared by carbonate extraction of Golgi membranes; see above) were solubilized in 0.1% SDS, 0.1 M NaHCO<sub>3</sub>, pH 8.3, 0.5 M NaCl (binding buffer), incubated overnight at 4°C with agitation, and clarified by

centrifugation at 10,000 g. Cyanogen bromide activated Sepharose, washed with 1 mM HCl, was added to the clarified GMPs at a final protein concentration of 3.4 mg/ml matrix. The Sepharose slurry was incubated overnight at 4°C with constant mixing. The matrix was subsequently incubated with 0.1% SDS, 0.2 M glycine, pH 8.0, for 2 h at 4°C, and then alternately washed for three separate rounds with binding buffer and 0.1% SDS, 0.1 M NaC<sub>2</sub>H<sub>3</sub>O<sub>2</sub>, pH 4.0. The matrix was mock eluted with 0.1% SDS, 0.1 M glycine, pH 2.8, 0.5 M NaCl (elution buffer) before equilibration in PBS (0.1% SDS). The efficiency of conjugation of GMPs to the matrix was judged to be 96.2%. Anti-GMP polyclonal antibodies were precipitated with ammonium sulfate, dialyzed against equilibration buffer and recycled over 2 ml of the GMP-Sepharose matrix at 4°C. After extensive washing with equilibration buffer, adsorbed antibodies were eluted in 0.5-ml fractions with 0.1% SDS, 0.1 M glycine, pH 2.8, 150 mM NaCl, and adjusted to pH 7.0 with 1 M Tris, pH 8.8. Affinity-purified antisera were pooled, concentrated, and assayed for protein content. Antisera was quantitated in ELISA as described using 0.7  $\mu$ g/ml final antibody concentration (Cleves et al., 1991b). Immunofluorescent colocalization was performed as previously described (Cleves et al., 1991b) with the following modifications. Briefly, formaldehyde fixed spheroplasts expressing KEX2 on a multicopy plasmid were deposited onto coverslips by a 5-min centrifugation at 1,000 g in a Cytospin 2 centrifuge (Cytospin 2; Shandon Inc., Sewickley, PA). Cells were subsequently immobilized on the cover slips by a 6-min incubation in ice-cold methanol, permeabilized by a 30–60-s rinse in ice-cold acetone, treated with 1% BSA (blocking buffer), and incubated with rabbit anti-KEX2p and mouse anti-GMP antibodies in blocking buffer containing 0.05% Tween 20 at concentrations of 13 and 23  $\mu$ g/ml, respectively. Spheroplasts were washed extensively in blocking buffer and subsequently incubated with FITC-conjugated goat anti-rabbit and Texas red-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch Labs., Inc., West Grove, PA) for 2 h at a concentration of 30  $\mu$ g/ml each. After extensive washing in blocking buffer, cells were visualized on a Zeiss Axioplan microscope equipped for fluorescence with UV filters and an HBO 50-W mercury lamp.

### Determination of Golgi Membrane Phospholipid Content

Purified Golgi membranes were prepared from the appropriate yeast cultures (1 liter) by two successive equilibrium sorbitol gradient centrifugations of the 100,000 g pellet fraction (P100) essentially as described by Cleves et al. (1991b) with the modification that a self-forming 30–80% equilibrium gradient was used to provide an initial fractionation of the P100. Cultures were grown to mid-logarithmic growth phase in YPD at 25°C, and shifted to YP (0.1% glucose medium) for an additional 3 h. Cells were then poisoned by the sequential addition of cycloheximide and NaN<sub>3</sub>, and further fractionated exactly as previously described (Cleves et al., 1991b). Cultures challenged with a temperature shift to 37°C were transferred to a prewarmed water bath 75 min before cycloheximide and NaN<sub>3</sub> addition. This period of temperature shift was more than sufficient to impose the *sec14-1<sup>ts</sup>* defect, a defect that is rapidly manifested upon temperature shift to 37°C (Bankaitis et al., 1989). Golgi membranes prepared by this procedure were routinely enriched some 185–200-fold over crude lysate. In cases where the *sec14-1<sup>ts</sup>* secretory block was imposed in otherwise wild-type

cells, the dysfunctional Golgi membranes were enriched some 150-fold over crude lysate (data not shown).

Phospholipids were extracted from purified Golgi fractions by organic extraction using 5 ml of  $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{BHT}$  (2:1:0.0005%). After gentle centrifugation to separate aqueous and organic phases, the organic phase was collected and washed 3 $\times$  with 2 ml of 10% methanol per wash to remove the excess sorbitol left over from the equilibrium gradients. The washed organic phase was subsequently dried under  $\text{N}_2$  gas, resuspended in 60  $\mu\text{l}$  of  $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{BHT}$  (2:1:0.0005%), and resolved by two-dimensional Whatman SG81 paper chromatography. First dimension solvent was  $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{NH}_4\text{OH}/\text{H}_2\text{O}$  (22:9:1:0.26) and second dimension solvent was  $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{CH}_3\text{COOH}/\text{H}_2\text{O}$  (8:1:1.25:0.25). Resolved lipids were stained with a 0.0012% solution of rhodamine 6G and visualized by illumination under UV light (Christie, 1987). The individual phospholipid spots were excised, the phospholipids eluted in  $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{BHT}$  (2:1:0.0005%), the phospholipid eluates dried under gentle vacuum with heat (65°C), and phospholipid content was quantitated by assay for total phosphorus by the method of Ames (1966).

For determination of the total Golgi glycerophospholipid content as a function of Golgi protein, Golgi fractions were purified from the appropriate yeast strains by the method described by Cleves et al. (1991b), and an aliquot of the purified Golgi fraction was used to determine protein by the BCA assay (see above). The washed organic extract of the remaining portion of the Golgi fractions was dried under gentle vacuum with heating (65°C), and total Golgi glycerophospholipid was quantitated by assay for phosphorus in the unresolved Golgi glycerophospholipid fraction by the method of Ames (1966).

### Radiolabeling of Phospholipids with [methyl- $^{14}\text{C}$ ]Methionine

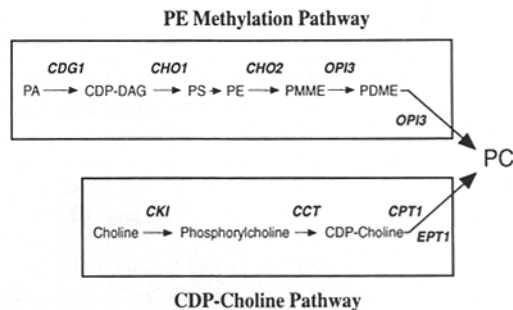
Pulse labeling of the appropriate yeast strains was performed on cultures grown to mid-logarithmic growth phase ( $\text{OD}_{600} = 0.8$ ) in the synthetic medium of Klig et al. (1985) and incubated with L-[methyl- $^{14}\text{C}$ ]methionine (1  $\mu\text{Ci}/\text{ml}$ ) for 30 min at 25°C with shaking. A small amount of unlabeled methionine was added to the culture simultaneously with the radiolabel to a final concentration of 7.5  $\mu\text{g}/\text{ml}$ . Incorporation of label into cells was measured by removing one-tenth of the culture, collection of cells from that culture aliquot onto 0.5  $\mu\text{M}$  glass fiber filters, washing of the filters 4 $\times$  with 50 vol of water per wash, and resuspension of the dried filters in scintillation fluid for counting. Phospholipids were extracted from the remainder of the culture as described above. The [methyl- $^{14}\text{C}$ ]methionine-labeled phospholipids were resolved by one-dimensional paper chromatography on Whatman SG81 paper treated as described above with  $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{NH}_4\text{OH}/\text{H}_2\text{O}$  (22:9:1:0.26) as the solvent. Quantitation of individual phospholipid species was by scintillation counting.

## Results

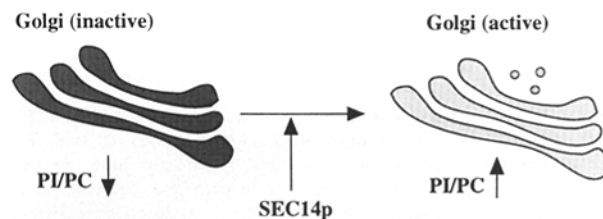
### A Subset of "Bypass SEC14p" Mutations Affect CDP-Choline Pathway Function

Disruption of structural genes for enzymes of the CDP-choline pathway (e.g., *CKI* and *CPTI*; see Fig. 1 A) effects bypass of the normally essential SEC14p requirement for Golgi secretory function (Cleves et al., 1989, 1991b). Mutation of any one of at least five other genes (i.e., *BSR2*, *BSR3*, *SAC1*, *BSD1*, and *BSD2*) is also sufficient to relieve yeast cells of their SEC14p requirement. To determine if inactivation of CDP-choline pathway function is a common feature of the "bypass SEC14p" alleles of the remaining five genes we have thus far identified, representative mutants were subjected to a [ $^{14}\text{C}$ ]choline radiolabeling regimen and analyzed for radiolabeled PC content. As shown by the data in Fig. 2, the *bsd1*, *bsd2*, and *sac1* mutants were all as proficient as the wild-type strain in incorporation of [ $^{14}\text{C}$ ]choline into PC. Thus, the corresponding wild-type gene products were not required for efficient synthesis of PC via the CDP-choline pathway in yeast. By contrast, the *bsr2-5* and *bsr3-2* mutations resulted in marked defects in [ $^{14}\text{C}$ ]choline incorpora-

A



B

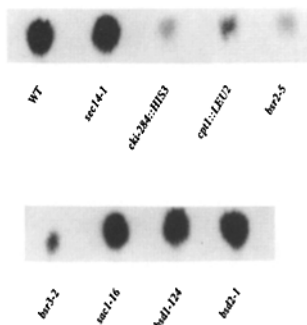


**Figure 1.** Pathways for PC biosynthesis in yeast and the PI/PC hypothesis for SEC14p function in vivo. (A) The two pathways for phosphatidylcholine synthesis in yeast are shown. Genes encoding the structural enzymes of each pathway are indicated at the site of action of their respective gene products. Phosphatidic acid (PA); CDP-diacylglycerol (CDP-DAG); phosphatidylserine (PS); phosphatidylethanolamine (PE); phosphatidylmonomethylethanolamine (PMME); phosphatidyltrimethylethanolamine (PDME); and phosphatidylcholine (PC). (B) The PI/PC hypothesis posits that SEC14p maintains an appropriately elevated PI/PC ratio in yeast Golgi membranes, a ratio that is proposed to be a critical determinant of yeast Golgi secretory competence (Cleves et al., 1991a,b).

tion into PC (Fig. 2), and the magnitude of these defects was at least as great as those exhibited by mutants harboring individual disruptions of structural genes for enzymes of the CDP-choline pathway (i.e., *cki-284::HIS3* and *cpt1::LEU2*, respectively). These [ $^{14}\text{C}$ ]choline incorporation data, when coupled with genetic data that indicate these *bsr2* and *bsr3* alleles to be recessive mutations (Cleves et al., 1991b), identify the *BSR2* and *BSR3* gene products as being required for CDP-choline pathway function in vivo. A combination of [ $^{14}\text{C}$ ]choline tracer experiments and integrative genetic mapping data have identified *BSR2* as the structural gene for the enzyme cholinephosphate cytidylyltransferase (CCTase) that catalyzes the second reaction of the CDP-choline PC biosynthetic pathway (Fig. 1 A), whereas *BSR3* encodes an activity that appears to influence expression of active CCTase in vivo (manuscript in preparation). These data distinguish two general classes of "bypass SEC14p" mutants; those that are deficient in CDP-choline pathway function (*cki*, *bsr2*, *bsr3*, *cpt1*), and those that are not (*bsd1*, *bsd2*, and *sac1*). The latter class of "bypass SEC14p" mutants will be treated in detail elsewhere.

### Bulk Membrane Phospholipid Profiles in Inositol- and Choline-containing Medium

The PI/PC hypothesis predicts that the relative PI/PC con-



**Figure 2.** Incorporation of choline into PC. The indicated strains were grown to mid-logarithmic growth phase in medium containing inositol (1 mM) but lacking choline. Cells were pulse radiolabeled for 20 min with [<sup>14</sup>C]choline (1 μCi/ml), collected by filtration, and washed to remove any unincorporated radiolabel. Glycerophospholipids were subsequently extracted

from the washed cells and resolved by one-dimensional paper chromatography as described in Materials and Methods. The various loads were normalized to incorporation of [<sup>14</sup>C]choline into cells (i.e., filtration-resistant material). Each lane represents glycerophospholipid extracted from 750,000 cpm [<sup>14</sup>C]choline incorporated into cells. Strains used were: *WT*, CTY182; *sec14-1<sup>ts</sup>*, CTY1-1A; *cki-284::HIS3*, CTY392; *cpt1::LEU2*, CTY434; *bsr2-5*, CTY105; *bsr3-2*, CTY156; *sac1-16*, CTY10; *bsd1-124*, CTY124; *bsd2-1*, CTY31. The complete genotypes of the strains employed are given in Table I.

content of Golgi membranes is directly dependent on SEC14p activity (Cleves et al., 1991a,b). Possible mechanisms of SEC14p bypass by inactivation of the CDP-choline pathway include: (a) a wholesale alteration in bulk membrane PL composition in such mutants; and (b) a local effect on the PC

content of Golgi membranes (Cleves et al., 1991a). To investigate the former possibility, we employed [<sup>32</sup>P]-orthophosphate (<sup>32</sup>P<sub>i</sub>) radiolabeling methods to quantitate the effects of "bypass SEC14p" mutations on general phospholipid metabolism in yeast grown in the presence of inositol and choline. Under pulse-radiolabeling conditions (30 min), the radiolabeled PL profile measured for wild-type cells consisted of 42% PI, 19% PC, 17% phosphatidylserine (PS), and 14% phosphatidylethanolamine (PE) (Table II). We infer the relative rates at which each PL species was synthesized during the pulse by the percentage of <sup>32</sup>P<sub>i</sub> incorporation into each individual PL species. A relative comparison of PI with respect to PC yielded a PI/PC ratio of 2.19 (Table II). Under these same experimental conditions, the isogenic *sec14-1<sup>ts</sup>* strain exhibited a bulk membrane radiolabeled PL profile and an efficiency of incorporation into glycerophospholipid, on a per cell basis, that was essentially indistinguishable from that of the wild-type strain (PI/PC ratio = 1.95; Table II). Thus, the *sec14-1<sup>ts</sup>* allele did not exert a measurable effect on bulk yeast membrane PL biosynthetic rates. In marked contrast, the *cki-284::HIS3*, *bsr2-5*, *bsr3-2*, and *cpt1::LEU2* mutants exhibited substantially reduced rates of bulk membrane PC synthesis. These strains incorporated only 5, 3, 5, and 3% of total lipid <sup>32</sup>P<sub>i</sub> into PC, respectively, even though these strains exhibited efficiencies of <sup>32</sup>P<sub>i</sub> incorporation into glycerophospholipid that were comparable to those exhibited by wild-type and *sec14-1<sup>ts</sup>* strains (Table II). These reductions in PC biosynthetic rate were observed

**Table II. Pulse and Steady-state Phospholipid Profiles of Yeast Grown in I<sup>+</sup>C<sup>+</sup> Media**

Strain	I <sup>+</sup> C <sup>+</sup> Radiolabeling					
	PI	PS	Pulse		cpm/10 <sup>5</sup> cells	PI/PC
			PC	PE		
	%	%	%	%		
<i>WT</i>	42.4 ± 5	17.2 ± 3	19.3 ± 4	13.5 ± 2	189 ± 11	2.20
<i>sec14-1</i>	42.0 ± 6	13.8 ± 2	21.6 ± 4	14.9 ± 4	183 ± 9	1.94
<i>cki-284::HIS3</i>	43.9 ± 5	16.3 ± 4	5.0 ± 3	20.5 ± 4	171 ± 9	8.78
<i>bsr2-5</i>	39.2 ± 3	19.5 ± 4	2.7 ± 1	25.2 ± 5	237 ± 15	14.52
<i>bsr3-2</i>	40.8 ± 4	15.6 ± 3	5.0 ± 2	23.9 ± 4	177 ± 10	8.16
<i>cpt1::LEU2</i>	46.1 ± 8	21.6 ± 5	2.9 ± 4	19.4 ± 3	171 ± 10	15.90
	Steady State					
	PI	PS	PC	PE		PI/PC
<i>WT</i>	24.2 ± 2 (112 ± 8)	9.3 ± 1 (46 ± 3)	43.5 ± 2 (186 ± 10)	17.0 ± 1 (79 ± 3)		0.56
<i>sec14-1</i>	23.8 ± 1 (114 ± 9)	8.4 ± 1 (46 ± 4)	45.4 ± 2 (187 ± 7)	18.4 ± 2 (84 ± 6)		0.52
<i>cki-284::HIS3</i>	24.6 ± 2 (120 ± 10)	7.0 ± 2 (41 ± 2)	41.0 ± 3 (187 ± 8)	17.3 ± 3 (65 ± 2)		0.60
<i>bsr2-5</i>	41.2 ± 3 (145 ± 8)	9.8 ± 1 (36 ± 3)	30.1 ± 2 (122 ± 11)	7.7 ± 5 (52 ± 6)		1.37
<i>bsr3-2</i>	30.2 ± 2 (114 ± 7)	9.4 ± 1 (30 ± 2)	41.6 ± 1 (133 ± 7)	13.9 ± 2 (54 ± 4)		0.73
<i>cpt1::LEU2</i>	21.7 ± 1 (107 ± 4)	8.6 ± 2 (40 ± 4)	39.6 ± 4 (175 ± 9)	13.1 ± 3 (64 ± 4)		0.55

Pulse and steady-state phospholipid profiles of yeast grown in I<sup>+</sup>C<sup>+</sup> media. For pulse-radiolabeling experiments, the indicated strains were grown to mid-logarithmic growth phase at 25°C in medium containing inositol and choline chloride (1 mM each) and pulse-radiolabeled with [<sup>32</sup>P]orthophosphate (10 μCi/ml) for 30 min. For steady-state radiolabeling experiments, the indicated strains were grown for five to six generations at 25°C in media containing *myo*-inositol and choline chloride (1 mM, each) supplemented with [<sup>32</sup>P]orthophosphate to 10 μCi/ml. Glycerophospholipids were extracted, resolved, and quantitated by liquid scintillation counting as described in Materials and Methods. In the pulse-radiolabeling experiments, the relative rates of synthesis for each individual phospholipid were inferred from the relative proportion of label recovered in each phospholipid species, and the mole percentage of label recovered in each individual phospholipid species is presented in the % PL column. Rates of total phospholipid synthesis were inferred from the incorporation of [<sup>32</sup>P]orthophosphate into phospholipid and these data are expressed as cpm recovered in the total phospholipid extract per 10<sup>5</sup> cells. PI/PC ratio was determined as the quotient of cpm recovered in PI and PC for each individual strain.

For steady-state radiolabeling experiments, phospholipid data for each strain are expressed both as the relative mole percentage of label recovered in each individual phospholipid species and, in parentheses, as cpm recovered for each phospholipid species per 10<sup>6</sup> viable cells. PI/PC ratio was determined as the quotient of cpm recovered in PI and in PC for each individual strain. Values presented represent averages from at least three independent trials. Strains used were: *WT*, CTY182; *sec14-1<sup>ts</sup>*, CTY1-1A; *cki-284::HIS3*, CTY392; *cpt1::LEU2*, CTY434; *bsr2-5*, CTY105; *bsr3-2*, CTY156; *sac1-16*, CTY10; *bsd1-124*, CTY124; *bsd2-1*, CTY31. The complete genotypes of these strains are given in Table I.

without significant deviation in the rates of PI and PS synthesis, although radiolabeled PE levels were somewhat elevated in these strains relative to wild type. Consequently, the bulk PI/PC ratios for these mutants were dramatically increased relative to the PI/PC ratio of the wild-type strain (Table II). On the basis of these data, we conclude that the alterations in phospholipid synthesis described above reflect specific defects in synthesis of a particular lipid species, and not a general defect in lipid synthesis per se.

Under a steady-state radiolabeling regimen, PC, PI, PS, and PE constituted some 45, 24, 9, and 17% of total membrane PL in wild-type and *secl4-1<sup>ts</sup>* strains (Table II). The corresponding PI/PC ratios were 0.56 and 0.52 for these strains, respectively. However, the steady-state PL profiles obtained for the *cki-284::HIS3*, *bsr3-2*, and the *cpt1::LEU2* "bypass SEC14p" mutants were similar to those of the wild-type and *secl4-1<sup>ts</sup>* strains, as were the corresponding steady-state PI/PC ratios (Table II). Moreover, the steady-state efficiencies of <sup>32</sup>P<sub>i</sub> incorporation into each lipid species were comparable for all of the strains used (Table II). Thus, the PC biosynthetic defects observed in the pulse experiments for these CDP-choline pathway-deficient strains were largely corrected at steady-state. Nevertheless, the *bsr2-5* mutant still exhibited a significant reduction in the steady-state PC content of bulk membranes in the face of a substantially normal efficiency of <sup>32</sup>P<sub>i</sub> incorporation into glycerolipid. PC constituted only 30% of total PL in this strain whereas 45% of total PL in the wild-type strain was PC. This reduction in PC, when coupled with a significant increase in bulk PI content (41% of total PL) in the *bsr2-5* mutant, yielded a bulk PI/PC ratio of 1.37; a ratio some 2.6-fold higher than that of wild-type membranes (Table II).

These data demonstrate that CDP-choline pathway dysfunction resulted in a clear reduction in the relative rate of PC biosynthesis, as inferred by the specific reduction in the rates of incorporation of labeled precursors into PC, and effected dramatic increases in the radiolabeled PI/PC ratios of bulk membranes when cells were grown in I<sup>+</sup>C<sup>+</sup> medium. With the exception of the *bsr2-5* mutant, these PL imbalances were largely corrected at steady-state. Moreover, since inactivation of the CDP-choline pathway reduced the rate of PC biosynthesis four- to sevenfold, these data demonstrate that the <sup>32</sup>P<sub>i</sub> pulse-radiolabeling regimen predominantly measured the rate of PC synthesis via the CDP-choline pathway when cells were grown in I<sup>+</sup>C<sup>+</sup> medium.

#### **The Choline Salvage Activity of the CDP-Choline Pathway Is Required for the Maintenance of Normal Cellular PC Biosynthetic Rates**

CDP-choline pathway dysfunction effects a bypass of SEC14p in choline-free medium but removal of choline from the medium does not affect the cellular requirement for SEC14p (Cleves et al., 1991b). Under choline-free growth conditions, the CDP-choline pathway is relegated to a salvage activity that can reincorporate the choline liberated by PC turnover back into newly synthesized PC, but is not capable of sustaining net cellular PC biosynthesis. Thus, we assessed the contribution of CDP-choline pathway activity to PL biosynthetic rates and bulk membrane PL composition when cells were cultured in inositol- and choline-free medium (I<sup>-</sup>C<sup>-</sup>). The data are shown in Table III. When wild-type

yeast were grown in choline-free medium at 25°C, PI, PS, PC, and PE represented some 11, 20, 13, and 15% of total <sup>32</sup>P<sub>i</sub>-labeled PL, respectively, when a 30-min pulse-radiolabeling regimen was followed. The PI/PC ratio of these membranes was 0.84. The pulse profile of PLs recovered from the isogenic *secl4-1<sup>ts</sup>* mutant was very similar to that measured in wild-type cells, and the corresponding PI/PC ratio and efficiency of incorporation of radiolabel into glycerophospholipid was also similar (Table III). Thus, the *secl4-1<sup>ts</sup>* allele did not exert any measurable effect on bulk PL biosynthetic rates when cells were grown in I<sup>-</sup>C<sup>-</sup> medium. Disruption of CDP-choline pathway function in the *cki-284::HIS3*, *bsr2-5*, *bsr3-2*, and *cpt1::LEU2* mutants resulted in dramatic reductions in incorporation of <sup>32</sup>P<sub>i</sub> into PC. In no case did PC content exceed 5% of total labeled PL, even though the efficiencies of incorporation of radiolabel into glycerolipid were not significantly affected in these mutants (Table III). Moreover, these mutants displayed significantly elevated PI/PC ratios ranging from 1.58 for the *cki-284::HIS3* strain to 10.15 for the *bsr2-5* strain.

Bulk membranes prepared from wild-type cells grown in I<sup>-</sup>C<sup>-</sup> medium exhibited 40% PC, 12% PI, 10% PS, and 13% PE at steady-state (Table III). The corresponding PI/PC ratio was 0.30. Again, the *secl4-1<sup>ts</sup>*, *cki-284::HIS3*, and *cpt1::LEU2* mutants each exhibited nearly normal steady-state PL profiles, efficiencies of incorporation of <sup>32</sup>P<sub>i</sub> into glycerolipid, and PI/PC ratios. Both the *bsr2-5* and *bsr3-2* mutants, however, exhibited significant alterations in their PL profiles. In the former case, the PC content (37%) was similar to that of wild type while the PI content (22%) was considerably higher, leading to a steady-state PI/PC ratio (0.58) that was nearly double that of the wild-type strain (0.30). Likewise, the bulk membranes prepared from the *bsr3-2* strain displayed increased steady-state levels of PI and an elevated PI/PC ratio, yet neither the *bsr2-5* strain nor the *bsr3-2* strain exhibited significant alterations in their ability to incorporate radiolabel into glycerolipid (Table III). We have repeated these steady-state analyses for all of these mutants (*cpt1* mutants excluded) in I<sup>-</sup>C<sup>+</sup> medium and have found that the bulk PL compositions were essentially the same as the corresponding compositions measured when these strains were grown in I<sup>-</sup>C<sup>-</sup> medium (data not shown). Thus, the alterations in bulk PL composition measured for *bsr2-5* and *bsr3-2* mutants grown in the absence of inositol were independent of the choline content of the growth medium.

These data demonstrate that CDP-choline pathway activity was required for the maintenance of normal bulk PL biosynthetic rates and, at least in the case of the *bsr2* and *bsr3* mutants, for the steady-state maintenance of normal PI and PC levels in bulk yeast membranes even when cells were grown in choline-free medium; i.e., a condition where the CDP-choline pathway is a choline salvage activity that cannot contribute net PC synthesis to the yeast cell. With respect to the former point, the finding that inactivation of the CDP-choline pathway resulted in an approximately threefold decrease in the rate of PC biosynthesis in cells grown in I<sup>-</sup>C<sup>-</sup> medium relative to wild-type cells reveals that PC synthesis via the choline salvage activity of the CDP-choline pathway is a significant contributor to PC biosynthetic rates in yeast grown under choline-free conditions.

Table III. Pulse and Steady-state Phospholipid Profiles of Yeast Grown in I<sup>-</sup>C<sup>-</sup> Media

Strain	I <sup>-</sup> C <sup>-</sup> Radiolabeling						
	PI	PS	PC	Pulse	PE	cpm/10 <sup>5</sup> cells	PI/PC
	%	%	%	%	%		
<i>WT</i>	10.7 ± 2	20.0 ± 5	12.8 ± 3	15.3 ± 4	211 ± 24	0.84	
<i>sec14-1</i>	12.0 ± 3	16.2 ± 4	13.3 ± 4	14.8 ± 4	189 ± 28	0.90	
<i>cki-284::HIS3</i>	8.5 ± 1	24.2 ± 5	5.4 ± 2	21.4 ± 6	196 ± 16	1.57	
<i>bsr2-5</i>	18.1 ± 4	23.7 ± 5	1.8 ± 1	21.8 ± 5	230 ± 15	10.15	
<i>bsr3-2</i>	10.9 ± 2	29.6 ± 6	2.1 ± 1	24.3 ± 6	182 ± 14	5.19	
<i>cpt1::LEU2</i>	15.5 ± 5	29.4 ± 5	4.5 ± 2	14.9 ± 3	169 ± 23	3.44	

	Steady State					
	PI	PS	PC	PE	PI/PC	
<i>WT</i>	12.0 ± 2 (86 ± 4)	10.2 ± 2 (64 ± 3)	40.1 ± 3 (243 ± 9)	13.1 ± 2 (92 ± 5)	0.30	
<i>sec14-1</i>	14.4 ± 2 (72 ± 5)	9.4 ± 2 (60 ± 2)	43.6 ± 2 (226 ± 8)	14.4 ± 1 (84 ± 3)	0.33	
<i>cki-284::HIS3</i>	12.5 ± 1 (68 ± 4)	9.8 ± 1 (54 ± 4)	46.5 ± 4 (244 ± 10)	16.6 ± 2 (83 ± 4)	0.27	
<i>bsr2-5</i>	21.5 ± 2 (117 ± 11)	8.8 ± 1 (56 ± 3)	36.9 ± 3 (187 ± 7)	19.7 ± 3 (86 ± 5)	0.58	
<i>bsr3-2</i>	25.4 ± 2 (87 ± 7)	9.8 ± 1 (39 ± 5)	39.4 ± 2 (144 ± 6)	17.3 ± 2 (70 ± 4)	0.65	
<i>cpt1::LEU2</i>	13.8 ± 1 (72 ± 6)	10.3 ± 3 (53 ± 4)	43.1 ± 4 (224 ± 8)	19.2 ± 2 (86 ± 3)	0.22	

Pulse and steady-state phospholipid profiles of yeast grown in I<sup>-</sup>C<sup>-</sup> medium. For pulse-radiolabeling experiments, the indicated strains were grown to mid-logarithmic growth phase at 25°C in inositol and choline-free medium and pulse-radiolabeled with [<sup>32</sup>P]orthophosphate (10 μCi/ml) for 30 min. For steady-state radiolabeling experiments, the indicated strains were grown for five to six generations at 25°C in inositol and choline-free medium supplemented with [<sup>32</sup>P]orthophosphate to 10 μCi/ml. Glycerophospholipids were extracted, resolved, and quantitated by liquid scintillation counting as described in Materials and Methods. In the pulse-radiolabeling experiments, the relative rates of synthesis for each individual phospholipid were inferred from the relative proportion of label recovered in each phospholipid species, and the mole percentage of label recovered in each individual phospholipid species is presented in the % PL column. Rates of total phospholipid synthesis were inferred from the incorporation of [<sup>32</sup>P]orthophosphate into phospholipid and these data are expressed as cpm recovered in the total phospholipid extract per 10<sup>5</sup> cells. PI/PC ratio was determined as the quotient of cpm recovered in PI and PC for each individual strain. For steady-state radiolabeling experiments, phospholipid data for each strain are expressed both as the relative mole percentage of label recovered in each individual phospholipid species and, in parentheses, as cpm recovered for each phospholipid species per 10<sup>6</sup> viable cells. PI/PC ratio was determined as the quotient of cpm recovered in PI and in PC for each individual strain. Values presented represent averages from at least three independent trials. Strains used were: *WT*, CTY182; *sec14-1*<sup>u</sup>, CTY1-1A; *cki-284::HIS3*, CTY392; *cpt1::LEU2*, CTY434; *bsr2-5*, CTY105; *bsr3-2*, CTY156; *sacl-16*, CTY10; *bsd1-124*, CTY124; *bsd2-1*, CTY31. The complete genotypes of these strains are given in Table I.

### *cpt1* and *ept1* Mutations Are Distinguished by Their Effects on PC Biosynthesis via the CDP-Choline Pathway In Vivo

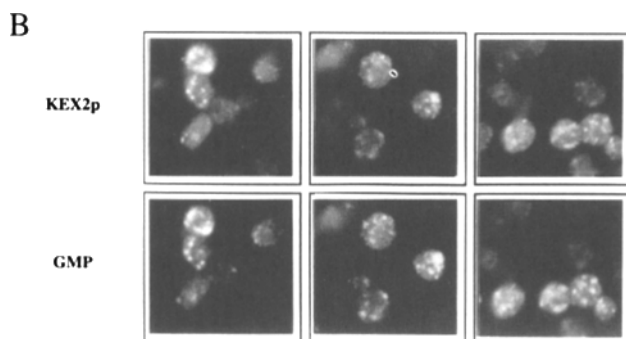
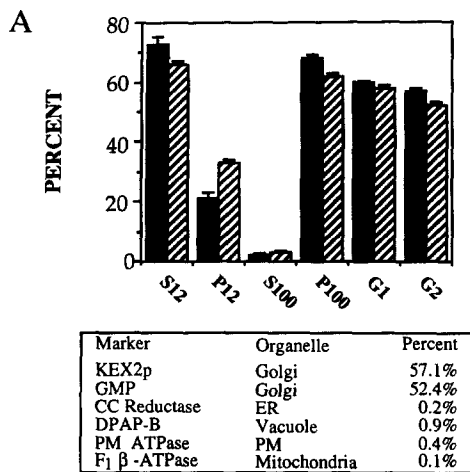
The *EPT1* and *CPT1* gene products are ethanolamine- and choline-phosphotransferases, respectively, that are each able to efficiently catalyze the ultimate reaction for PC biosynthesis via the CDP-choline pathway in vitro (Hjelmstad and Bell, 1987; Hjelmstad and Bell, 1988). Nevertheless, *cpt1* disruption mutations effect a bypass of SEC14p whereas *ept1* disruption mutations do not (Cleves et al., 1991b). As a common feature of CDP-choline pathway defects that bypass SEC14p is a reduction in the cellular rate of PC biosynthesis (see above), we tested the effect of an *ept1::URA3* allele on the efficiency of PC synthesis in vivo. Whereas *cpt1::LEU2* mutants were clearly defective in the incorporation of [<sup>14</sup>C]choline into PC, and suffered markedly reduced rates of PC biosynthesis when cells were grown either in I<sup>+</sup>C<sup>+</sup> or I<sup>-</sup>C<sup>-</sup> medium (Fig. 2, Tables II and III), *ept1::URA3* mutants were not significantly compromised for PC biosynthesis. Yeast strains carrying *ept1::URA3* exhibited normal rates of [<sup>14</sup>C]choline incorporation into PC, and the bulk membrane PL profiles of *ept1::URA3* cells were indistinguishable from those of wild type in both <sup>32</sup>P<sub>i</sub> pulse radiolabeling and steady-state radiolabeling experiments that were performed with cells growing in either I<sup>+</sup>C<sup>+</sup> or I<sup>-</sup>C<sup>-</sup> medium (data not shown). These results indicate that, even though the *CPT1* and *EPT1* gene products exhibit functional redundancy in vitro, these enzymes are not functionally re-

dundant with respect to PC synthesis in vivo. Moreover, these data further strengthen the relationship between the ability of yeast cells to synthesize PC via the CDP-choline pathway and the essential SEC14p requirement of yeast cells for Golgi secretory function and cell viability.

### Characterization of Yeast Golgi Membrane Purity

The PI/PC hypothesis predicts that the relative PI/PC content of Golgi membranes is directly dependent on SEC14p activity (Cleves et al., 1991a,b). To directly monitor the phospholipid content of yeast Golgi membranes, we developed a Golgi enrichment procedure to obtain sufficient quantities of highly purified Golgi membranes for biochemical analysis. This method involves serial differential centrifugation steps followed by two successive equilibrium gradient centrifugation steps (Cleves et al., 1991b; see Materials and Methods). However, since meaningful analyses of Golgi membrane PL content require a very high degree of Golgi membrane purity, we characterized in detail the purity of the Golgi membranes recovered from lysates prepared from a wild-type yeast strain (CTY182; see Table I). Characterization of the recoveries of marker proteins that identify defined organelles in the most highly enriched Golgi fractions demonstrated that this procedure yielded an excellent recovery of KEX2p (ca. 60% of total), a resident integral membrane protein of the yeast Golgi (Fig. 3 A). These Golgi fractions represented a 185–200-fold enrichment of KEX2p over total cell lysate under all of the various experimental condi-





**Figure 3.** Purity of yeast Golgi membrane preparations. (A) The distribution of the Golgi marker enzyme, KEX2p, and of Golgi membrane proteins (GMPs) through the Golgi enrichment procedure is shown (top). The percentage of total starting KEX2p (■) and of GMP signal (▨) recovered in the 12,000 g supernatant (S12), 12,000 g pellet (P12), 100,000 g supernatant (S100), 100,000 g pellet (P100), G1, and G2 fractions. G1 represents the peak KEX2p fraction pool obtained after resolution of the P100 in a 30–80% sorbitol equilibrium gradient (see Materials and Methods). G2 represents the most highly purified Golgi membrane fraction and was obtained by resolution of the G1 fraction in a 40–65% sorbitol equilibrium gradient, again as previously described (Cleves et al., 1991b). Also tabulated is the percent recovery of defined markers from the most purified Golgi fractions (G2) relative to total marker present in the unfractionated cell-free lysate (bottom). The proportion of total starting signal for Golgi, endoplasmic reticulum (ER), vacuole, plasma membrane (PM), and mitochondrial marker enzymes present in the G2 fraction is given. Marker enzymes were determined as described in Materials and Methods. (B) Colocalization of GMPs and the Golgi marker KEX2p. To visualize KEX2p, fixed, and permeabilized cells of the KEX2p-overproducer strain CTY234 were incubated with affinity-purified polyclonal rabbit anti-KEX2p serum and Texas red sulfonyl chloride (TRSC)-conjugated donkey anti-rabbit IgG. GMPs were visualized with primary anti-GMP mouse polyclonal antibodies and developed with FITC-conjugated sheep anti-mouse IgG. Displayed here are representative panels of cells that were analyzed via this double-label immunofluorescence regimen. The top panels record the KEX2p profile while the bottom panels record the corresponding GMP profile. Appropriate controls demonstrated the specificity of the antisera and the spectral separation of the fluorochromes under the photographic conditions employed (not shown). Other technical details are presented in the Materials and Methods.

tions used in this study and were substantially free of markers diagnostic of endoplasmic reticulum (ER), mitochondrial, vacuolar, and plasma membranes.

To gain an independent measure of the purity of these Golgi membranes, we raised a polyspecific polyclonal antiserum in mice against bulk integral membrane proteins of the most highly enriched Golgi fractions (see Materials and Methods). We will henceforth refer to this population of integral membrane proteins as GMPs (Golgi Integral Membrane Proteins). This anti-GMP serum recognized 7–10 major polypeptides, as judged by immunoblotting experiments, and revealed a polypeptide pattern that was similar to that observed when GMPs were displayed by SDS-PAGE and visualized by silver staining (manuscript in preparation). This antiserum was then used to compare the distribution of GMPs with that of KEX2p throughout the Golgi enrichment procedure, and to assess the degree of colocalization of GMPs with Golgi membranes (i.e., KEX2p) in double-label indirect immunofluorescence experiments. As shown by the data presented in Fig. 3 A, the distribution of the GMPs throughout the Golgi enrichment procedure was very similar to that of the Golgi marker KEX2p, and the yield of GMPs was comparable to that of KEX2p. The most highly enriched Golgi fraction contained 57 and 52% of the total KEX2p and GMPs, respectively (Fig. 3 A), yet we estimate this fraction contained only an approximately 0.3% recovery of total cellular glycerophospholipid (not shown).

For double-label indirect immunofluorescence experiments we employed strain CTY234, a KEX2p overproducing strain (Table I), as it has previously been shown that yeast bearing the *KEX2* gene on a multicopy plasmid exhibit normal localization of KEX2p, and that the amplified KEX2p staining can be readily detected without having to resort to antibody sandwich methods (Franzusoﬀ et al., 1991; Redding et al., 1991). Fig. 3 B shows representative GMP and KEX2p staining patterns that were observed in fixed yeast cells. Both the GMP and KEX2p staining profiles largely consisted of three to 15 randomly distributed punctate bodies per cell, and were very similar to the KEX2p staining profiles previously described (Cleves et al., 1991; Franzusoﬀ et al., 1991; Redding et al., 1991). Preimmune serum collected from the mouse from which the anti-GMP serum was derived gave no detectable signal in control immunofluorescence experiments (not shown). Further comparison of these localization profiles revealed a high coincidence of the punctate GMP and KEX2p signals. Finally, the lack of GMP-specific staining of cellular membranes that did not contain KEX2p was particularly noteworthy (Fig. 3 B). These collective biochemical fractionation and immunofluorescence localization data indicate a high level of purity of the isolated Golgi membranes. We note, however, that we were able to achieve this requisite purity of Golgi membranes only when cells were cultured in YPD and not when cells were grown in minimal media (not shown).

#### **The PC Content of Yeast Golgi Membranes Varies as a Direct Function of SEC14p Activity**

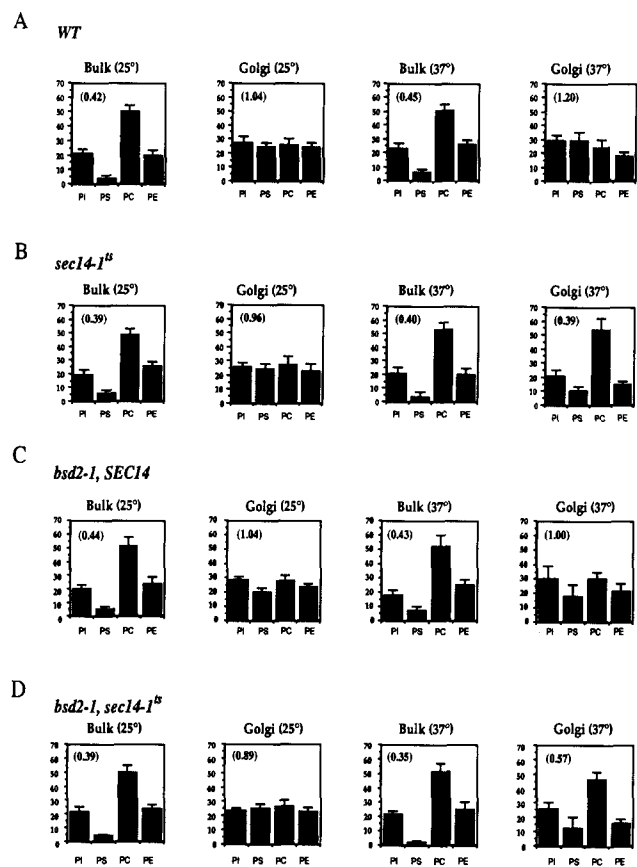
To directly test the dependence of Golgi membrane PL composition on SEC14p activity, we analyzed the PL composition of purified Golgi membranes under various conditions



of SEC14p function and dysfunction. As shown by the data in Fig. 4 A, the wild-type PL composition of Golgi membranes was distinct from that of bulk membranes. Whereas bulk membranes consisted of 50, 21, 4, and 20% of PC, PI, PS, and PE, respectively, Golgi membranes were composed of nearly equivalent mole percentages of each of these PL species. These values translated into a PI/PC ratio of 0.42 for bulk membranes and 1.04 for Golgi membranes, a 2.5-fold difference in PI/PC ratio between these two membrane preparations. Moreover, neither the bulk nor the Golgi membrane PL compositions of wild-type cells were affected by shift to 37°C (Fig. 4A).

A distinguishing feature of *sec14-1<sup>ts</sup>* strains is that these mutants exhibit a dramatic exaggeration of the Golgi complex upon shift to 37°C. This phenotype is considered to reflect continued membrane and protein flow into the Golgi complex from the early stages of the secretory pathway without the normal balance of SEC14p-dependent membrane and protein exit from the Golgi into the latest stages of the secretory pathway (Novick et al., 1980). Thus, the yeast Golgi PL composition of *sec14-1<sup>ts</sup>* mutants should be significantly altered as a secondary consequence of imposition of the *sec14-1<sup>ts</sup>* secretory block. The data presented in Fig. 4 B indicate that both the bulk membrane (PI/PC = 0.39) and Golgi membrane (PI/PC = 0.96) PL compositions of *sec14-1<sup>ts</sup>* cells grown at 25°C were very similar to the corresponding membrane PL compositions of wild-type cells. However, although shift of *sec14-1<sup>ts</sup>* mutants to 37°C did not significantly alter the bulk membrane PL composition in these mutants, this temperature shift effected a dramatic change in the *sec14-1<sup>ts</sup>* Golgi PL profile (Fig. 4 B). The Golgi PL composition became similar to the bulk membrane composition (PI/PC = 0.39). We note that, under these restrictive conditions, the fractionation properties of the *sec14-1<sup>ts</sup>* Golgi changed slightly in that the compartment became denser. Nevertheless, we were able to enrich for *sec14-1<sup>ts</sup>* Golgi membranes some 150-fold over crude lysate (not shown). These data indicate that the *sec14-1<sup>ts</sup>* mutation did not affect either bulk or Golgi membrane PL compositions at the permissive temperature of 25°C, nor did *sec14-1<sup>ts</sup>* affect bulk membrane PL composition at the restrictive temperature of 37°C. Rather, the *sec14-1<sup>ts</sup>* defect became apparent only at the level of Golgi membranes. In addition, this dramatic alteration of Golgi PL profile by imposition of the *sec14-1<sup>ts</sup>* block was not some completely nonspecific consequence of secretory pathway dysfunction or general growth arrest as it was not observed when either the *sec11<sup>ts</sup>* or the *sec15<sup>ts</sup>* secretory blocks were imposed on the corresponding mutants by temperature shift to 37°C (not shown). The *sec11<sup>ts</sup>* mutation causes defects in signal peptide cleavage from secretory glycoproteins and delays their export from the ER (Schauer et al., 1985; Bohni et al., 1988). The *sec15<sup>ts</sup>* mutation blocks fusion of Golgi-derived secretory vesicles to the plasma membrane (Novick et al., 1980).

To measure Golgi membrane PL content as a direct function of SEC14p activity, it was necessary to uncouple Golgi secretory function from its usual SEC14p requirement because of the Golgi PL alterations that likely occur simply as an indirect consequence of imposition of the *sec14-1<sup>ts</sup>* secretory block (see above). We achieved this end by using strain CTY31, a *sec14-1<sup>ts</sup>* strain that also carries the *bsd2-1* allele (see Table I). The *bsd2-1* allele is a “bypass SEC14p” muta-



**Figure 4.** Phospholipid composition of bulk yeast membranes and Golgi membranes. For these experiments, yeast strains with the indicated relevant genotypes were grown to mid-logarithmic phase at 25°C in YPD medium and, where indicated, shifted to 37°C for 75 min prior to harvesting of cells to induce dysfunction of the *sec14-1<sup>ts</sup>* gene product (Sec14p<sup>ts</sup>). Samples were removed from each culture prior to generation of spheroplasts and determination of bulk membrane PL composition as described in the Materials and Methods. Golgi membranes were subsequently purified from the remainder of the culture, and individual PL species were resolved by two-dimensional paper chromatography and quantitated by phosphorus assay (see Materials and Methods). Values presented are the mole percentage of total glycerolipid phosphorus represented by each individual phospholipid species ( $n = 3$ ). Quantitation of the major phospholipids of yeast, that is phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylinositol (PI), is indicated for both bulk cell membranes and purified Golgi membranes. Minor species such as phosphatidic acid (PA), cardiolipin (CL), phosphatidylmonomethylethanolamine (PMME), and phosphatidyl dimethylethanolamine (PDME) collectively constituted less than 1% of total phospholipid. Inset values reflect the average PI/PC ratio displayed by each membrane preparation. Strains used for each panel were: (A) CTY182; (B) CTY1-1A; (C) CTY479; (D) CTY31 (see Table I).

tion that relieves Golgi secretory function from its usual SEC14p requirement (Cleves et al., 1991b), but does so without effecting wholesale alterations in bulk membrane PL composition or content. The steady-state bulk membrane PL compositions of the *bsd2-1, SEC14* strain (Fig. 4 C) and the *bsd2-1, sec14-1<sup>ts</sup>* strain (Fig. 4 D) were indistinguishable from those of the wild-type strain (Fig. 4 A). Golgi membranes prepared from the *bsd2-1, SEC14* strain exhibited a

wild-type Golgi PL profile (PI/PC = 1.0) regardless of whether the strain had been subjected to the 37°C temperature shift, or not (Fig. 4, compare *C* with *A*). A normal Golgi membrane PL profile was also measured for the *bsd2-1*, *secl4-1<sup>ts</sup>* mutant (PI/PC = 0.9) when it was uniformly incubated at a temperature (25°C) permissive for Sec14p<sup>ts</sup> (Fig. 4, compare *D* with *A*). Thus, the *bsd2-1* mutation alone did not measurably alter relative Golgi membrane PL composition either at 25° or 37°C under SEC14p-proficient conditions. Furthermore, wild-type Golgi membranes were estimated to contain 43.6 (± 2.6) nmoles glycerophospholipid per mg Golgi protein after shift to 37°C, while *bsd2-1*, *SEC14* Golgi membranes were measured to contain 39.7 (± 2.4) nmoles glycerolipid per mg protein under the same culture conditions. Thus, the *bsd2-1* mutation alone also did not measurably alter the Golgi membrane PL: protein ratio at 37°C under SEC14p-proficient conditions. However, inactivation of Sec14p<sup>ts</sup> by temperature shift to 37°C resulted in nearly a doubling of the relative Golgi membrane PC content in the *bsd2-1*, *secl4-1<sup>ts</sup>* strain (PI/PC = 0.57; Fig. 4, compare *D* to *A* and *C*); despite the fact that this mutant is entirely proficient in Golgi secretory function and cell growth at this normally restrictive temperature (Cleves et al., 1991b). Moreover, the marked increase in Golgi PC content in the *bsd2-1*, *secl4-1<sup>ts</sup>* strain was not accompanied by dramatic alterations in the relative Golgi content of the other PLs, particularly PS. This was in contrast to the more general alterations in Golgi PL profile recorded upon imposition of the *secl4-1<sup>ts</sup>* Golgi secretory block (Fig. 4 *B*).

The observed alterations in Golgi phospholipid composition of *bsd2-1*, *secl4-1<sup>ts</sup>* strain upon shift to 37°C, relative to that of the *bsd2-1*, *SEC14* strain, could result from one of two effects: (a) a specific increase in net Golgi PC content in the *bsd2-1*, *secl4-1<sup>ts</sup>* strain; or (b) no change in Golgi membrane PC levels in the *bsd2-1*, *secl4-1<sup>ts</sup>* strain superimposed upon a general reduction in the Golgi membrane content of the other glycerophospholipids. To distinguish between these two possibilities, we compared the glycerophospholipid:protein ratios of Golgi membranes prepared from wild-type, *bsd2-1*, *secl4-1<sup>ts</sup>* and *bsd2-1*, *SEC14* yeast strains (see Materials and Methods). The first possibility predicted an increase in the Golgi phospholipid: protein ratio in the *bsd2-1*, *secl4-1<sup>ts</sup>* strain relative to the wild-type and *bsd2-1*, *SEC14* strains, while the latter predicted a relative decrease in the Golgi phospholipid: protein ratio in the *bsd2-1*, *secl4-1<sup>ts</sup>* strain. Golgi prepared from the *bsd2-1*, *secl4-1<sup>ts</sup>* strain after shift to 37°C, was estimated to contain 53.9 (± 1.9) nmoles of glycerophospholipid per mg Golgi protein, as compared to the corresponding wild type and *bsd2-1*, *SEC14* values of 43.6 (± 2.6) and 39.7 (± 2.4) nmoles glycerophospholipid per mg protein, respectively (see above). This increase reflected approximately a 24% increase in total Golgi glycerophospholipid (specifically PC) as a result of SEC14p dysfunction. Analysis of each PL species as a function of protein indicated that Golgi membranes of a *bsd2-1*, *SEC14* strain shifted to 37°C exhibited approximately 11.6 nmoles PC per mg Golgi protein, while the isogenic *bsd2-1*, *secl4-1<sup>ts</sup>* strain cultured under the identical conditions exhibited a Golgi membrane content of approximately 23.5 nmoles PC per mg protein. Thus, the collective data indicate that, while the nmoles PI, PS, and PE per mg Golgi protein present in *bsd2-1*, *SEC14* and *bsd2-1*, *secl4-1<sup>ts</sup>* Golgi membranes upon shift

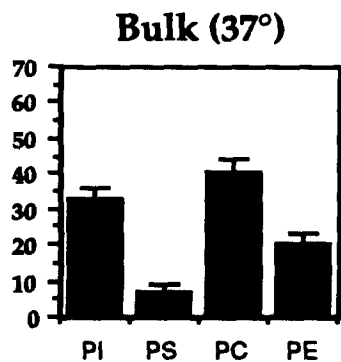
to 37°C remained relatively unchanged, the nmoles PC per mg Golgi protein in *bsd2-1*, *secl4-1<sup>ts</sup>* Golgi membranes effectively doubled relative to the nmoles PC per mg protein in *bsd2-1*, *SEC14* Golgi membranes upon shift to 37°C. This increase in the amount of PC relative to the amount of Golgi protein was reflected as an increase in total Golgi membrane phospholipid. These collective results indicate that the relative increase in Golgi PC content observed as a direct consequence of SEC14p dysfunction reflected a specific net increase in Golgi PC and not a condition of constant PC levels superimposed upon reductions in Golgi PI, PS, and PE. We conclude from these biochemical data that SEC14p functions to maintain a reduced PC content in yeast Golgi membranes.

### **CDP-Choline Pathway Dysfunction Restores Wild-type Golgi PL Composition to SEC14p-deficient Golgi Membranes**

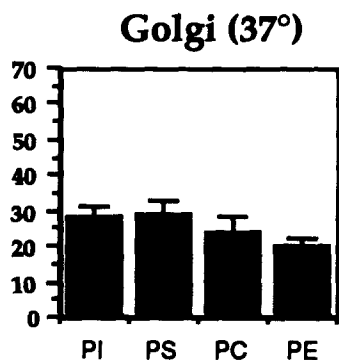
"Bypass SEC14p" mutants carrying the *secl4-1<sup>ts</sup>* allele and either the *cki-284::HIS3* or *cpt1::LEU2* alleles exhibited essentially wild-type bulk membrane profiles at steady-state, regardless of whether these strains were grown in I<sup>+</sup>C<sup>+</sup> or I<sup>-</sup>C<sup>-</sup> medium (see above). As a result, it was of interest to determine whether these mutations eliminated the increase in Golgi PC measured under conditions of SEC14p dysfunction. The *secl4-1<sup>ts</sup>*, *cki-284::HIS3* mutant was used for these studies. This mutant was grown at 25°C in YPD and shifted to 37°C, prior to harvesting of cells, to inactivate Sec14p<sup>ts</sup> (see Materials and Methods). When grown in the YPD medium that we must employ for preparative purification of Golgi membranes, the *secl4-1<sup>ts</sup>*, *cki-284::HIS3* strain exhibited a steady-state bulk membrane PL profile that consisted of 33% PI, 40% PC, 7% PS, and 20% PE, respectively (Fig. 5 *A*). Although this bulk membrane PL composition was similar to that exhibited by the isogenic *secl4-1<sup>ts</sup>* strain analyzed under these same conditions (Fig. 4 *B*), the profiles exhibited subtle differences. In particular, the *cki-284::HIS3* strain exhibited some elevation in bulk membrane PI and a minor decrease in bulk membrane PC relative to the isogenic *CKI* derivative (compare Figs. 5 *A* and 4 *B*). Nonetheless, the Golgi membranes prepared from the *secl4-1<sup>ts</sup>*, *cki-284::HIS3* strain exhibited a wild-type Golgi PL profile (compare Fig. 5 *B* with Fig. 4 *A*), and not the dramatically altered PL profile measured for either the isogenic *secl4-1<sup>ts</sup>*, *CKI* strain (compare Figs. 5 *B* and 4 *B*) or the congenic *secl4-1<sup>ts</sup>*, *bsd2-1* strain grown under these same conditions (compare Figs. 5 *B* and 4 *D*). Moreover, the phospholipid content of *secl4-1<sup>ts</sup>*, *cki-284::HIS3* and wild-type Golgi membranes was measured to be similar when the corresponding strains were cultured under the described conditions (45.3 ± 3.1 and 43.6 ± 2.6 nmole phospholipid per μg protein, respectively). Thus, *cki-284::HIS3* not only suppressed the lethality associated with SEC14p defects, but it concomitantly suppressed the relative increase in Golgi PC measured under conditions of SEC14p dysfunction.

Further confirmation of these results was obtained from analysis of the bulk and Golgi PL compositions of *secl4-1<sup>ts</sup>*, *bsd2-1*, *cki-284::HIS3* triple mutants. These triple mutants exhibited essentially wild-type bulk and Golgi membrane PI and PC profiles (22 ± 3 and 24 ± 2 mole% Golgi phospholipid, respectively) as well as Golgi phospholipid content per unit protein under Sec14p<sup>ts</sup>-deficient conditions (38.2 ± 3.3

A

*cki-284::HIS3, sec14-1<sup>ts</sup>*

B

*cki-284::HIS3, sec14-1<sup>ts</sup>*

**Figure 5.** Golgi membrane phospholipid composition of *sec14-1<sup>ts</sup>, cki* double mutants. (A) The bulk membrane composition of CTY392 (*sec14-1<sup>ts</sup>, cki-284::HIS3*) is shown. Glycerophospholipids were extracted from a culture of CTY392, which had been shifted to 37°C for 75 min, and were resolved and quantitated as described in Materials and Methods and in the legend to Fig. 3. (B) The PL composition of Golgi membranes prepared from strain CTY392 after a 75 min shift to 37°C is shown. Glycerophospholipids were extracted from purified Golgi membranes and the mole percentage of each glycerolipid is indicated ( $n = 3$ ).

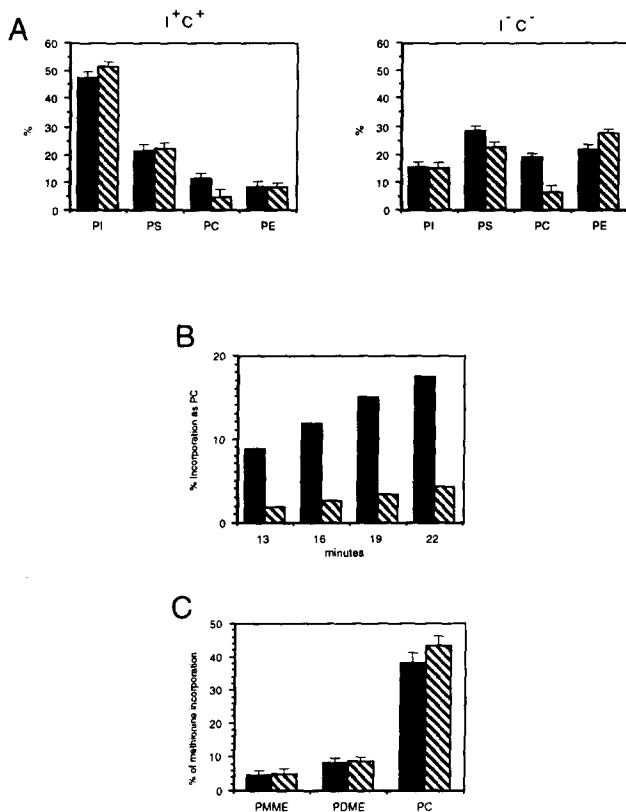
nmole glycerophospholipid per mg protein as compared to  $43.6 \pm 2.6$  nmoles glycerophospholipid per mg protein for wild-type Golgi membranes; see above), indicating that *cki-284::HIS3* eliminated the marked increase in Golgi PC normally observed in *sec14-1<sup>ts</sup>, bsd2-1* strains (see Fig. 4 D). These data suggest that PC synthesis via the CDP-choline pathway accounts for the net increase in Golgi PC that results from SEC14p dysfunction.

#### **SEC14p Overproduction Specifically Depresses PC Synthesis via the CDP-Choline Pathway**

The collective genetic and biochemical data can be interpreted to indicate either that: (a) SEC14p removes excess PC from Golgi membranes in vivo by virtue of its PL-TP activ-

ity; or (b) that SEC14p serves to inhibit PC synthesis via the CDP-choline pathway in vivo. In the case of the latter scenario, such a regulation may largely represent a local effect on PC biosynthesis exerted at the level of Golgi membranes. A prediction of that latter model, which distinguishes it from the PL transfer model, is that overproduction of SEC14p in yeast might result in a more general inhibition of cellular CDP-choline pathway activity in vivo. To test this hypothesis, we overproduced SEC14p in yeast by increasing *SEC14* gene dosage and estimated PC biosynthetic rates in such a SEC14p overproducing strain. *SEC14* gene dosage was increased by introducing a YEp(*SEC14*) plasmid into an appropriate yeast strain. The YEp(*SEC14*) derivative strain routinely overproduced SEC14p some 5–10-fold as judged by quantitative immunoprecipitation, immunoblotting, and in vitro PI-transfer assays (not shown). As demonstrated by the  $^{32}\text{P}_i$  pulse-radiolabeling data in Fig. 6 A, a comparison of the bulk membrane PI, PS, and PE profiles of a wild-type yeast strain and its isogenic SEC14p-overproducing derivative demonstrated that these profiles were very similar, regardless of whether the strains were cultured in I<sup>+</sup>C<sup>+</sup> or I<sup>-</sup>C<sup>-</sup> media. However, the pulse-radiolabeled bulk membrane PC content of the SEC14p-overproducing strain was substantially reduced (2.5- to 3-fold) relative to that of the wild-type strain; irrespective of whether the corresponding yeast strains were grown in I<sup>+</sup>C<sup>+</sup> or I<sup>-</sup>C<sup>-</sup> media. Indeed, the magnitudes of the SEC14p-overproduction-dependent reduction in bulk membrane PC content of cells grown in I<sup>+</sup>C<sup>+</sup> and I<sup>-</sup>C<sup>-</sup> media were very similar to those observed for *cki-284::HIS3, bsr2, bsr3,* and *cpt1::LEU2* yeast strains cultured under the corresponding growth conditions (see above). The steady-state PL compositions of the wild-type and SEC14p overproducer strains were indistinguishable, indicating that the SEC14p-overproduction mediated effect on bulk PC content was corrected at the level of steady-state (not shown). These data indicate that overproduction of SEC14p resulted in a general depression in the rate of cellular PC biosynthesis.

Since the rate of PC biosynthesis inferred from the pulse-radiolabeling experiments reflects predominantly PC synthesis via the CDP-choline pathway (see above), the reduction in pulse-radiolabeled bulk membrane PC content measured in the SEC14p-overproducing strain likely represented a specific reduction in the rate of PC biosynthesis via the CDP-choline pathway. To more directly assess CDP-choline pathway activity as a function of SEC14p overproduction, we measured the time course of [ $^{14}\text{C}$ ]choline incorporation into PC in a wild-type and SEC14p-overproducing yeast strain as a function of cell number. Representative data are presented in Fig. 6 B. At all of the time points taken, the wild-type strain exhibited an approximately fourfold greater incorporation of [ $^{14}\text{C}$ ]choline into PC than did the isogenic SEC14p-overproducing strain. Thus, we concluded that SEC14p-overproduction effected a significant reduction in the rate of PC biosynthesis via the CDP-choline pathway. Pulse-radiolabeling of bulk membrane PC with [*methy*- $^{14}\text{C}$ ] methionine, a specific measure of PC biosynthesis via the PE methylation pathway, did not reveal a reduction in the efficiency of incorporation of this label into bulk membrane PC for the SEC14p-overproducing strain relative to the wild-type strain (Fig. 6 C). These collective data suggest that overproduction of SEC14p effected a marked and specific



**Figure 6.** Overproduction of SEC14p inhibits the activity of the CDP-choline pathway. (A) Wild-type strain CTY525 (■) and the isogenic SEC14p overproducing strain CTY521 (▨) were grown to mid-logarithmic phase at 25°C in the indicated media and pulse-radiolabeled with [<sup>32</sup>P]orthophosphate (10 μCi/ml) for 30 min. Synthesis of the major PLs was evaluated as described in Materials and Methods. Values presented represent the average mole percentage of total lipid-extractable radiolabeled material (designated as % on the y-axis) associated with each lipid species (*n* = 3). (B) CTY525 (■) and CTY521 (▨) were grown in I<sup>-</sup>C<sup>-</sup> minimal medium and 2 OD<sub>600</sub> of cells were pulse-radiolabeled with [<sup>14</sup>C]choline (1 μCi/ml) for the indicated periods of time. Samples were removed from the labeling culture and evaluated for total uptake of label into cells while radiolabeled PLs were extracted from the remainder of the culture (see Materials and Methods). Values show the percentage of total label taken up by the cells which had been incorporated into PC in a representative experiment (determined as described in Materials and Methods). The magnitude of reduction in incorporation of label into PC observed for the SEC14 overproducing strain relative to the isogenic wild-type strain has been reproduced in four independent experiments. (C) For this experiment, strains CTY525 and CTY521 were grown in methionine-free I<sup>-</sup>C<sup>-</sup> medium to mid-log phase and pulse-radiolabeled with [methyl-<sup>14</sup>C] methionine for 20 min. The percentage of total label taken up by the cell which had been incorporated into PC (indicated on y-axis) was determined as described in Materials and Methods. These values represent the average of three independent trials.

reduction in the rate of PC biosynthesis via the CDP-choline pathway.

## Discussion

The PI/PC hypothesis for SEC14p function posits that SEC14p acts to maintain an appropriately elevated PI/PC ra-

tio in Golgi membranes (Cleves et al., 1989, 1991b). This hypothesis was proposed in an attempt to reconcile: (a) the findings that SEC14p is required for Golgi secretory function in vivo and exhibits PI/PC transfer activity in vitro; and (b) the genetic data demonstrating that disruption of structural genes for enzymes of the CDP-choline pathway effects bypass of the normally essential SEC14p requirement for Golgi secretory function and yeast cell viability (reviewed in Cleves et al., 1991a). The PI/PC hypothesis makes one basic experimental prediction; namely, that the PI/PC ratio in yeast Golgi membranes will decrease as a direct consequence of SEC14p dysfunction. The biochemical data reported here provide strong evidence to support the basic prediction of the PI/PC hypothesis, allow us to reconcile the puzzling issues of the inability of choline deprivation or disruption of *EPT1* to bypass SEC14p, and suggest a mechanism for how SEC14p stimulates Golgi secretory function in vivo.

Highly purified yeast Golgi membranes prepared under SEC14p-proficient conditions exhibited a unique PL composition; one that was substantially elevated in PS, slightly elevated in PI and PE, and substantially reduced in PC relative to bulk membranes (Fig. 4 A). Thus, yeast Golgi membranes exhibited an enrichment in acidic PLs (i.e., PI and PS) as has also been measured for other organelle membranes of the late secretory pathway (Zinser et al., 1991). Under conditions where Golgi secretory function was uncoupled from its usual SEC14p requirement by virtue of the *bsd2-1* "bypass SEC14p" allele, imposition of SEC14p dysfunction resulted in a nearly twofold increase in the relative PC content of yeast Golgi membranes (Fig. 4 D). With the important caveat that we do not know how *bsd2-1* effects bypass of SEC14p, other than this bypass does not appear to be mediated through substantial effects on bulk membrane or Golgi PL composition and content (Fig. 4, C and D), these data demonstrate that SEC14p functions to maintain the reduced PC content of wild-type Golgi membranes in vivo. We also noted that the PI levels of yeast Golgi membranes did not measurably vary as a function of SEC14p activity, suggesting that SEC14p is not involved in controlling PI levels in yeast Golgi membranes in vivo. This is an intriguing result as PI is the preferred SEC14p ligand in the in vitro PL transfer reaction (Daum and Paltauf, 1984).

Of the seven genes identified by "bypass SEC14p" mutations (i.e., *SAC1*, *BSD1*, *BSD2*, *CKI*, *CPT1*, *BSR2*, and *BSR3*), genetic, and biochemical data identified four of these to encode gene products that are required for CDP-choline pathway activity in vivo (Cleves et al., 1991b; Fig. 2). Inactivation of CDP-choline pathway activity by mutation of *CKI*, *CPT1*, *BSR2*, or *BSR3* uniformly resulted in a significant decrease in the rate of cellular PC biosynthesis, as inferred from <sup>32</sup>P<sub>i</sub> pulse-radiolabeling experiments, regardless of whether cells were grown in I<sup>+</sup>C<sup>+</sup> or I<sup>-</sup>C<sup>-</sup> medium (Tables II and III). A consequence of this depression in cellular PC biosynthetic rate was an increase in the corresponding PI/PC ratios of pulse-radiolabeled bulk membrane PLs. Moreover, the finding that PC synthesis via the CDP-choline pathway made significant contributions to the cellular rate of PC biosynthesis, even when cells were grown in choline-free medium, has profound ramifications for the specific organelles that exhibit such PC biosynthetic capability. If yeast Golgi membranes are engaged in PC biosynthesis via the CDP-choline pathway, as is the case in mammalian cells

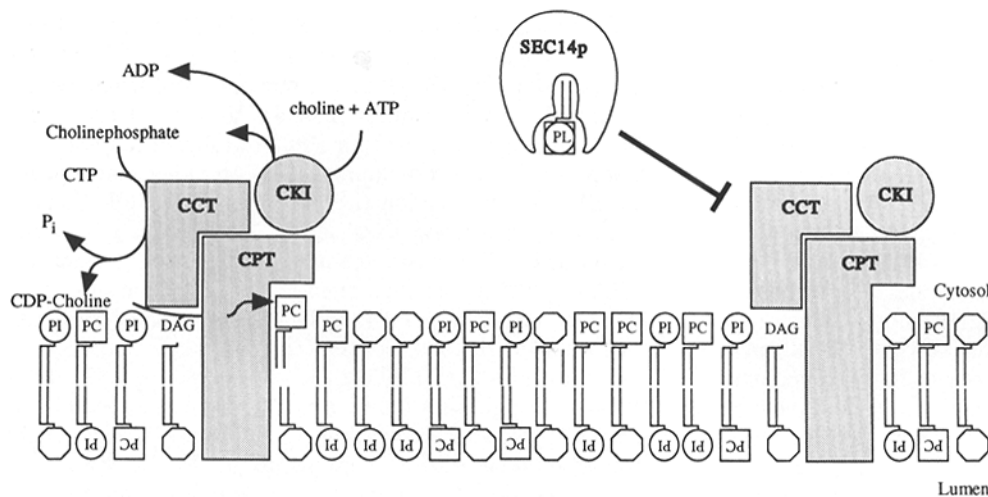
(Vance and Vance, 1988; reviewed in Van Meer, 1989), these membranes will experience significant net PC synthesis even under conditions where no net cellular PC synthesis is possible via the CDP-choline pathway. The idea that the yeast Golgi complex has such PC biosynthetic capability is supported by our finding that mutational inactivation of the CDP-choline pathway prevented, in SEC14p-deficient Golgi membranes, the near doubling of Golgi PC content that was measured as a primary effect of SEC14p dysfunction (Fig. 5 B). On the basis of these collective data, we predict that mutational inactivation of the CDP-choline pathway will manifest itself in a significant reduction in the relative Golgi membrane PC content of cells grown in I<sup>-</sup>C<sup>-</sup> medium, as was measured for cells cultured in the choline-replete YPD medium (Fig. 5 B). Unfortunately, technical difficulties associated with purifying Golgi membranes from cells grown in minimal medium have precluded these very important measurements. Finally, in contrast to the *CPTI* gene product, the *EPTI* gene product did not play a significant role in PC biosynthesis via the CDP-choline pathway in vivo (see above), irrespective of the shared ability of both of these gene products to efficiently catalyze the ultimate step in PC biosynthesis via the CDP-choline pathway in vitro (Hjelmstad and Bell, 1987, 1988). This finding fully accounts for why *cpt1::LEU2* is a "bypass SEC14p" mutation whereas *ept1::URA3* is not, and reinforces the genetic data that indicate SEC14p function to be tied to CDP-choline pathway activity in vivo (Cleves et al., 1991b).

How might SEC14p function interface with PC synthesis via the CDP-choline pathway? The genetic data demonstrating that disruption of structural genes for CDP-choline pathway enzymes obviates the normally essential cellular requirement for SEC14p can be interpreted to indicate that PC synthesis via the CDP-choline pathway is inherently toxic to Golgi secretory function and, therefore, cell viability. SEC14p would then, in principle, serve to detoxify the deleterious effect of CDP-choline pathway activity in vivo. Until now, we have considered SEC14p function in terms of its in vitro PI/PC transfer activity (Cleves et al., 1991a, b). By this view, SEC14p could be interpreted to effect net removal of PC from yeast Golgi membranes via its PC transfer activity. This interpretation is consistent with the data indicating that SEC14p exhibits PL-TP activity in vitro, that SEC14p functions to maintain a reduced PC content in yeast Golgi membranes, and that SEC14p is found both as a cytosolic species and a peripheral Golgi membrane protein (Bankaitis et al., 1989; Cleves et al., 1991b). However, the difficulties with this mechanism for SEC14p function are twofold: (a) it fails to offer an explanation for why PI is not a relevant substrate in vivo when PI (not PC) is the preferred SEC14p ligand in vitro; and (b) this model does not provide ready insight with respect to the relevance of the SEC14p requirement in the face of CDP-choline pathway activity, but its irrelevance with respect to PC synthesis via the PE methylation pathway (Cleves et al., 1991b; McGee et al., 1992). As there exists no in vivo evidence to indicate that any PL-TP genuinely functions in intracellular PL transport per se, the in vitro PL transfer activity must be viewed with proper caution. Indeed, it is clear that SEC14p does not play an essential in vivo role in the retrieval, by bulk transport, of PL from the late stages of the secretory pathway back to the ER (Cleves et al., 1991b); as had been proposed by Rothman (1990).

Our finding that overproduction of SEC14p resulted in a specific reduction in cellular PC synthesis via the CDP-choline pathway (Fig. 6), while not obviously consistent with a PL transfer mechanism for SEC14p function, does suggest an alternative mechanism for SEC14p function in vivo; one that posits a sensor function for SEC14p rather than a genuine PL transfer function (i.e., the catalysis of PL transfer from one membrane to a second biochemically distinct membrane). The basic features of a sensor model for SEC14p function are illustrated in Fig. 7. In that model we consider the various genetic and biochemical data to indicate that SEC14p serves to repress CDP-choline activity in yeast Golgi membranes. SEC14p is considered to exist in a dynamic equilibrium between Golgi membrane-bound and cytosolic states, thereby allowing SEC14p to continuously exchange bound PL for Golgi membrane PL. As a result, a peripheral SEC14p population is maintained whose bound PL component in effect reflects the Golgi membrane PI/PC content at any given time and can potentially be used as a signal through which a sensitive regulation of the local CDP-choline pathway activity in Golgi membranes could be achieved. For example, the PI- or PC-bound form of SEC14p could potentially serve as a specific allosteric repressor of Golgi PC synthesis via the CDP-choline pathway (Fig. 7). This model accounts for the available data concerning SEC14p function in vivo, including the localization of SEC14p to Golgi membranes and cytosol and the dramatic increase in Golgi membrane PC content by inactivation of SEC14p in the face of a functional CDP-choline pathway (Cleves et al., 1991b; Fig. 4 D). Moreover, this model provides an explanation for why suppression of SEC14p dysfunction is effected by inactivation of the CDP-choline pathway but not the PE methylation pathway for PC synthesis.

We also find the sensor model for SEC14p function in vivo to be attractive not only because it is consistent with the in vivo data, but because it reconciles a curious property of SEC14p-mediated PL transfer activity in vitro with SEC14p function in vivo. It has long been appreciated that many PL-TPs, SEC14p included, efficiently catalyze PL exchange reactions in vitro but are not efficient catalysts of net PL transfer reactions (Daum and Paltauf, 1984; Wirtz, 1991; Rueckert and Schmidt, 1991). This property is not immediately consistent with a role for SEC14p in limited intracellular PL transport reactions as net PL transfer by definition accomplishes work whereas PL exchange need not. The sensor model predicts that the functional reaction mode for SEC14p in vivo will be simple PL exchange, not genuine PL transport, and leaves open the possibility that PI could be a relevant ligand in a Golgi PI/PC sensing reaction (Fig. 7). Thus, the increase in Golgi PC that directly results from SEC14p dysfunction is proposed to reflect loss of a local SEC14p-dependent downregulation of PC synthesis via the CDP-choline pathway and not loss of SEC14p-mediated transport of PC from the Golgi. However, we must emphasize that the sensor model does not resolve all of the in vivo data. This model, in its basic form, suffers from its failure to offer a specific mechanism for why Golgi secretory function exhibits such a remarkable differential sensitivity to PC synthesis via each of these two PC biosynthetic pathways.

In summary, we have obtained biochemical data that demonstrate the in vivo function of SEC14p to be the maintenance of a reduced PC content in yeast Golgi membranes;



**Figure 7.** The sensor model. The sensor model proposes that Golgi membranes have PC biosynthetic capability via the CDP-choline pathway, and that SEC14p functions to specifically regulate PC synthesis via the CDP-choline pathway as a function of the PI, PC, or relative PI/PC content of yeast Golgi membranes. SEC14p is proposed to sample Golgi PI and/or PC composition by constantly exchanging bound PL for Golgi membrane PL, an activity that would manifest itself as an efficient PI/PC-TP exchange activity in vitro and an efficient sensing reaction in

vivo. A specific PL-bound isoform of SEC14p could then serve to, either directly or indirectly, regulate the rate of PC biosynthesis via the CDP-choline pathway in an allosteric manner. The genetic data indicating that loss of CDP-choline pathway function results in a "bypass SEC14p" phenotype suggests a negative regulatory role for SEC14p in such a process (Cleves et al., 1991b). The enzymes of the CDP-choline PC biosynthetic pathway are indicated: choline kinase (CKI), choline-phosphate cytidylyltransferase (CCT), and cholinephosphotransferase (CPT). This model satisfactorily accounts for the available in vivo data for SEC14p function without invoking a genuine PL transfer mechanism for SEC14p activity.

specifically a reduced contribution of PC synthesized via the CDP-choline pathway to the PC content of yeast Golgi membranes. We also demonstrate that PC synthesis via the CDP-choline pathway is a significant contributor to the rate of PC biosynthesis in cells, irrespective of whether this pathway contributes to net cellular PC synthesis or whether it is relegated to a choline salvage activity that cannot contribute to net cellular PC synthesis. The finding that disruption of CDP-choline pathway function eliminates the dramatic increase in Golgi PC that is a direct result of SEC14p dysfunction provides a biochemical basis for why mutational inactivation of the CDP-choline pathway results in bypass of the normally essential SEC14p requirement for Golgi secretory function and cell viability. Finally, we describe evidence consistent with a sensor mechanism for SEC14p function that accounts for most of the available data in a model that does not invoke a genuine PL transfer function for the SEC14p. This sensor model embraces a novel concept that may prove to be more widely applicable to PL transfer proteins that efficiently catalyze in vitro PL exchange reactions, but are inefficient catalysts of net PL transfer reactions.

What are the ramifications of these data for the PI/PC hypothesis? Although the data reported here highlight reduced PC biosynthesis via the CDP-choline pathway as being crucial for yeast Golgi secretory function, other data suggest PI metabolism may also play an important role. A role for inositol phospholipids, or their turnover, has been implicated in *sacl*-mediated bypass of SEC14p (Whitters et al., 1993). In this regard, we note that PC synthesis via the CDP-choline pathway (but not the methylation pathway) directly consumes diacylglycerol, a product of PL-turnover by the action of phospholipase C. Thus, the possibility must also be considered that: (a) the various "bypass SEC14p" mutations share the unifying property of elevating, or otherwise maintaining, Golgi diacylglycerol levels; and (b) that the "bypass SEC14p" effect of CDP-choline pathway dysfunction reflects a potentiation of a diacylglycerol-mediated signal transduction event

rather than a primary effect of PC content on Golgi secretory processes. Elucidation of the mechanisms by which *bsd1*, *bsd2*, and *sacl* mutations suppress SEC14p defects in the face of a functional CDP-choline pathway should allow us to discriminate whether diacylglycerol regulates some essential reaction required for protein secretion from the Golgi complex, or whether yeast Golgi membranes specifically require a reduced PC composition for the fulfillment of their essential secretory function.

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