Role of the Yeast Phosphatidylinositol/Phosphatidylcholine Transfer Protein (Sec14p) in Phosphatidylcholine Turnover and INO1 Regulation*

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In yeast, mutations in the CDP-choline pathway for phosphatidylcholine biosynthesis permit the cell to grow even when the SEC14 gene is completely deleted (Cleves, A., McGee, T., Whitters, E., Champion, K., Aitken, J., Dowhan, W., Goebi, M., and Bankaitis, V. (1991) Cell 64, 789–800). We report that strains carrying mutations in the CDP-choline pathway, such as cki1, exhibit a choline excretion phenotype due to production of choline during normal turnover of phosphatidylcholine. Cells carrying cki1 in combination with sec14ts, a temperature-sensitive allele in the gene encoding the phosphatidylinositol/phosphatidylcholine transporter, have a dramatically increased choline excretion phenotype when grown at the sec14ts-restrictive temperature. We show that the increased choline excretion in sec14ts cki1 cells is due to increased turnover of phosphatidylcholine via a mechanism consistent with phospholipase D-mediated turnover. We propose that the elevated rate of phosphatidylcholine turnover in sec14ts cki1 cells provides the metabolic condition that permits the secretory pathway to function when Sec14p is inactivated.

As phosphatidylcholine turnover increases in sec14ts cki1 cells shifted to the restrictive temperature, the INO1 gene (encoding inositol-1-phosphate synthase) is also derepressed, leading to an inositol excretion phenotype (Opi-). Misregulation of the INO1 gene has been observed in many strains with altered phospholipid metabolism, and the relationship between phosphatidylcholine turnover and regulation of INO1 and other coregulated genes of phospholipid biosynthesis is discussed.

Phospholipid transfer proteins capable of exchanging phospholipids between membrane bilayers in vitro have been extensively characterized, but the role of these proteins in vivo has been more difficult to establish (1–5). A breakthrough in this analysis occurred with the discovery that the product of the Saccharomyces cerevisiae SEC14† gene, which is required for the secretory process, encodes a PI/PC² transfer protein (5). Deletion of the yeast SEC14 gene in an otherwise wild type cell is lethal and inactivation of the SEC14 gene product in a temperature-sensitive (sec14ts) mutant leads to arrest of the secretory pathway at the late Golgi stage (6). Analysis of suppressors that permit the sec14ts mutant to grow at the restrictive temperature led to the discovery that mutations in the CDP-choline pathway for PC biosynthesis (Fig. 1) suppress the sec14 mutant phenotype, allowing wild type growth even in strains carrying a total deletion of the SEC14 gene (1).

The CDP-choline pathway, first described by Kennedy and Weiss (7), is one of two routes for synthesis of PC in eukaryotic cells, including yeast. The second route for synthesis of PC, originally described by Bremer and Greenberg (8), involves methylation of PE. Yeast cells can utilize either the CDP-choline or the PE methylation pathway, or a combination of the two, for net PC synthesis (9) (Fig. 1). Deletion of the genes in the CDP-choline pathway is not lethal in yeast and, indeed, appears to have little effect on growth (9, 10). It has been widely assumed that the CDP-choline pathway in yeast functions largely for the utilization of exogenous choline. However, recent studies have suggested that the CDP-choline pathway contributes substantially to PC biosynthesis even in the absence of exogenous choline (10–12). In the absence of exogenous choline, the yeast cell synthesizes PC predominantly via methylation of PE.

Conversely, yeast cells can survive the complete and simultaneous deletion of the genes encoding the two phospholipid methyltransferases that carry out the three-step conversion of PE to PC, provided choline is supplied in the growth medium (13).

However, deletions of genes encoding enzymes in either of these two pathways result in subtly different phenotypes. For example, the deletion of either of the phospholipid methyltransferases does not suppress the sec14 growth phenotype (1, 14). Such mutants are, however, unable to repress the INO1 gene (encoding inositol-1-phosphate synthase; see Fig. 1) in

† The structural genes are as follows: INO1, inositol-1-phosphate synthase; CHO1, PS synthase; PSD1, PS decarboxylase; CHO2 (PEM1), PE N-methyltransferase; OP3 (PEM2), phospholipid N-methyltransferase; CKI1, choline kinase; CCT1, choline phosphate-CTP cytidyltransferase; CPT1, sn-1,2-diacylglycerol choline phosphotransferase; SEC14, PI/PC transporter.

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response to exogenous inositol, unless PC biosynthesis is restored via the CDP-choline pathway (13, 15). And while the deletion of genes encoding enzymes of the CDP-choline pathway does not affect INO1 regulation in response to inositol (9), such mutants suppress the sec14 phenotype (1, 14).

The INO1 gene is the most highly regulated of a set of genes encoding enzymes of phospholipid biosynthesis that are subject to complex coordinate control. All these genes contain a conserved promoter element, UASINO, that includes within it the canonical binding site, CANNTG, for transcription factors of the basic helix-loop-helix class (16, 17). In the present report, we show that inactivation of the sec14 gene and, by extension, other co-regulated genes of phospholipid metabolism.

**EXPERIMENTAL PROCEDURES**

**Culture Conditions**—Yeast strains were maintained on YEPD medium (1% yeast extract, 2% Bactopeptone, 3% glucose). Chemically defined synthetic medium was prepared as described previously (9). Synthetic medium either lacked inositol (I`) or was supplemented with 75 μM inositol (I`) and/or 1 mM choline (C`).

**Yeast Transformation**—Yeast transformation was performed by the lithium acetate method (18) with minor modifications.

**Assay for Opi**—(Overproduction of Inositol) and Opc—(Overproduction of Choline) Phenotypes—To test for the Opi` phenotype (see Refs. 19 and 20 for a complete method description), strains were patched onto synthetic I` or I plus medium lacking choline (C`).

**Phospholipid Analysis**—To determine steady state phospholipid

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**Figure 1. Diagram of ³²P-H₃PO₄ and [¹⁴C]choline labeling routes in S. cerevisiae.** Labeled phosphorus (³²P) is represented by *P, and labeled choline ([¹⁴C]) is represented by ![Image of a diagram showing phosphorus and choline labeling routes in S. cerevisiae](http://www.emb.org). Intracellular water-soluble metabolites are shown in ovals (I-¹⁴C, inositol 1-phosphate; Glu-⁶⁻P, glucose 6-phosphate; I⁻¹⁴C, choline phosphate; CD⁻³⁻P⁻³⁻C, cytidine-diphosphate choline). Glycerophosphoinositol (Gro*PIns) is an extracellular metabolite, which can be taken up from the media. Lipids are shown in rectangles (DAG, diacylglycerol; *PA, phosphatidic acid; CD⁻³⁻P⁻DG, cytidine diphosphate-diacylglycerol; *PG⁻⁵⁻P, phosphatidylglycerol phosphate; *PG⁻⁵⁻P, phosphatidylglycerol; *PS, phosphatidylserine; *PI, phosphatidylinositol; *PI⁻¹⁴F⁻¹⁴P, phosphatidylinositol bisphosphate. Solid lines represent routes of metabolic conversion. Dashed lines indicate potential flux across the plasma membrane.
composition, strains were grown in I⁻ or I⁺ synthetic media containing 10 μCi of [³²P]orthophosphate/ml. The cultures were harvested in mid-logarithmic phase (A₅₉₅ = 0.4–0.6) after five to six generations of growth at the indicated temperature. Labeled lipids were extracted (25), individual phospholipid species were resolved by two-dimensional paper chromatography (26) and quantified by liquid scintillation counting. For phospholipid turnover experiments, cells were labeled as described above, harvested, washed twice in fresh media lacking labeled orthophosphate, and suspended at A₅₉₅ = 0.1 under the indicated culture conditions. Aliquots of these cultures were removed at indicated times, and phospholipids were analyzed. The rate of phospholipid synthesis in vivo was determined by pulse labeling the cells for 30 min with 50 μCi of [³²P]orthophosphate/ml as described by Kelley et al. (27), followed by lipid extraction and separation by paper chromatography, as described above. The amount of [³²P]phosphate incorporated into sphingolipids was determined by deacylating the extracted lipids and separating the sphingolipids by one-dimensional chromatography (28).

**Metabolic Labeling with [¹⁴C]Choline—**Strains were grown overnight at the indicated temperatures in I⁻ media containing 1 μCi/ml 1.2-[¹⁴C]choline at a concentration of 10 μM. Cultures were harvested during mid-logarithmic phase, washed twice in fresh non-radioactive media, and suspended at A₅₉₅ = 0.1 in I⁻ media. At the indicated time points, aliquots of the cultures were removed, and the cells were pelleted by centrifugation. The supernatant was saved as the “media” fraction. The cell pellet was processed as described (25) for the extraction of lipids with one addition; following treatment of the cell pellet with trichloroacetic acid to permeabilize the cell membrane, the supernatant and subsequent pellet washes were combined and saved as the “intracellular water-soluble fraction of the cell.” This fraction contained the vast majority of the intracellular water-soluble counts, as evidenced by the fact that >90% of the counts found in the only other cellular fraction (the lipid fraction) were shown by chromatography to be PC.

**Separation of Choline-containing Water-soluble Metabolites—**Cation exchange chromatography on Bio-Rex 70 resin (50–100 mesh) was used to separate quantitatively the 1.2-[¹⁴C]choline in the aqueous media washes or the trichloroacetic acid extracts from other water-soluble 1.2-[¹⁴C]choline-containing metabolites (29). The aqueous samples (3–5 ml) were neutralized with 1.0 M Tris buffer, pH 8.0, as necessary, and applied to a 1-ml Bio-Rex 70 column. The column was then washed with 5 ml of H₂O, followed by 10 ml of 50 mM glycine, 500 mM NaCl, pH 3.0, to elute the choline retained by the resin. The fractions were counted to determine the percentages of various metabolites present.

**RESULTS**

**Conditional Inositol and Choline Excretion Phenotype in Strains Carrying the sec14ts Allele in Combination with CDP-choline Pathway Mutations**—The overproduction of inositol (Opi⁻) phenotype (Fig. 2) is associated with misregulation of the INO1 gene (9, 16, 19). We report here a related choline excretion phenotype (Fig. 3), which we are designating as Opc⁻ (overproduction of choline). The Opi⁻ and Opc⁻ phenotypes of the strains used in this study are shown in Figs. 2 and 3 and are summarized in Table II. As reported previously (9, 30), strains carrying only mutations in the CDP-choline pathway exhibited no Opi⁻ phenotype. However, all of the CDP-choline pathway mutants exhibited the Opc⁻ phenotype to varying degrees. No Opi⁻ phenotype was observed for the strain carrying the sec14ts mutation (but no mutation in the CDP-choline pathway) at its permissive temperature, 25°C, or at 30°C (Table II). (This strain will be referred to from here on as sec14ts CKI1 (Table I) because most of the subsequent studies were done with the set of strains carrying combinations of the sec14ts and cki1 mutation.) A slight Opc⁻ phenotype was observed for the sec14ts CKI1 strain, but only in I⁻ media at 30°C.

At 30°C, a temperature that is still permissive for sec14ts, the double mutants containing sec14ts in combination with each of the CDP-choline pathway lesions (i.e. sec14ts cki1; sec14ts cct1; sec14ts cpt1) had an Opc⁻ phenotype somewhat stronger than the phenotype exhibited by strains carrying only the CDP-choline pathway mutation in question (Table II). At 25°C, none of these double mutant strains exhibited an Opi⁻
The increase in choline excretion at the restrictive temperature was reutilized via the CDP-choline pathway without escaping from the cells at the permissive temperature of 25 °C, a greater proportion of the label was associated with PC.

We found that incorporation of $[^{14}C]$choline into PC in SEC14 chi1 and sec14+ chi1 strains was approximately 13% of the wild type level, comparable to previous reports for chi1 strains (9, 31). Analysis of the chloroform-soluble label extract from all four strains labeled at 25 °C (i.e. wild type, sec14+ CK11, SEC14 chi1, and sec14+ chi1) demonstrated that >90% of the label was associated with PC.

To study PC turnover, $[^{14}C]$choline-labeled cells were shifted to unlabeled medium and the fate of the label was charted. The pattern of label transfer between the lipid-soluble pool, the intracellular water-soluble pool, and the medium following the shift to unlabeled medium is shown for three strains (wild type, SEC14 chi1, and sec14+ chi1) at 37 °C (Figs. 4 and 5). All four strains were tested in this fashion at 25 °C as well (data not shown); only two patterns of turnover were observed. The "wild type" pattern was exhibited by both wild type and sec14+ chi1 strains at 25 °C and by wild type at 37 °C (Fig. 4A). The "cki1" pattern was exhibited by both SEC14 chi1 and sec14+ chi1 strains at 25 °C and by the SEC14 chi1 strain at 37 °C (Fig. 4B).

In strains exhibiting the "wild type" pattern of choline metabolism (i.e. the wild type strain at both 25 °C and 37 °C and sec14+ chi1 only at the permissive temperature of 25 °C), the cells at the time of transfer to unlabeled medium had a significant amount (19–29%) of their cellular $^{14}C$ label in a water-soluble intracellular pool (Fig. 4A). The remaining cellular label was lipid-associated. A small amount of label (2–7%) was associated with extracellular free choline at 25 °C; however, at 37 °C, this extracellular label declined to a few percent, and the label appeared in the lipid-associated pool, which was shown to be greater than 90% PC. Very little label (10% or less of the total label) appeared in the medium throughout the 6-h chase (Figs. 4A and 5). In the case of wild type cells, the extracellular label that did appear was not in the form of free choline (Fig. 5). Most likely, it is glycerophosphocholine, known to be excreted by wild type yeast cells (32).

In strains exhibiting the "cki1" pattern of choline metabolism (i.e. SEC14 chi1 at both 25 °C and 37 °C, and sec14+ chi1 only at the permissive temperature of 25 °C), a greater proportion of total cellular label was present in the lipid fraction at the start of the experiment (Fig. 4B). The water-soluble intracellular pool was 12% or less of total label. In the SEC14 chi1 strain at both temperatures, as well as the sec14+ chi1 strain at 25 °C, there was little or no transfer of label from the intracellular soluble pool to PC following the shift to unlabeled medium. However, steady loss of label from PC (about 7% in 3 h) was observed, with a corresponding appearance of label associated with free choline in the growth medium (shown for the SEC14 chi1 strain at 37 °C in Figs. 4B and 5).

Finally, a third and very distinctive pattern of PC turnover was exhibited by the sec14+ chi1 strain at 37 °C (Fig. 4C). At 37 °C, the sec14+ chi1 strain had an initial label distribution at
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Fig. 4. Phosphatidylcholine turnover in wild type, SEC14 cki1, and sec14c cki1 strains at 37 °C. Strains were grown in I- medium containing 1 μCi/ml [14C]choline to mid-logarithmic phase. At time zero, cells were harvested, washed, and recultured in non-radioactive I- medium. At the indicated times, aliquots of the culture were analyzed for radioactivity. Total radioactivity incorporated into the sec14c cki1 and SEC14 cki1 strains was only 13% of the incorporation in wild type, consistent with previous reports (see Footnote 3). Data are expressed as a percentage of total label recovered in each fraction at each time point after the shift to unlabeled medium: culture medium (○), cellular water-soluble fraction (■), and cellular chloroform-soluble fraction (△). Data are the average of two independent experiments.

Fig. 5. Choline accumulation in the medium of wild type, SEC14 cki1, and sec14c cki1 cultures at 37 °C. These experiments were performed as described in Fig. 4. [14C]Choline was separated from other [14C]choline-containing metabolites by cation exchange chromatography as described under “Experimental Procedures.” Data are presented as the percentage of total radioactivity recovered at each time point in each strain and are the average of two independent experiments. The overall incorporation of [14C]choline into the two strains carrying the cki1 mutation (i.e. SEC14 cki1 and sec14c cki1) was only 13% of the wild type strain (SEC14 CKI1).

the time of shift to unlabeled medium similar to the SEC14 cki1 strain, but it lost label from PC much more rapidly (54% in the first 3 h). Again, the label appeared in the medium as free choline (Figs. 4C and 5). This finding is consistent with the strong Opc phenotype associated with the sec14c cki1 strain at 37 °C (Fig. 3). We also examined the loss of 14C label from PC in the sec14c cki1 and SEC14 cki1 strains when the cells were labeled with [14C]choline to steady-state at 25 °C and were shifted to unlabeled medium at 37 °C. The SEC14 cki1 strain, which had identical patterns of turnover at 25 °C and 37 °C (Fig. 4B), not surprisingly showed a similar labeling pattern when shifted from 25 °C to 37 °C. However, when shifted to 37 °C after labeling at 25 °C, the sec14c cki1 strain, which had different labeling patterns at the two temperatures, exhibited the pattern of 14C loss from PC observed when the labeling and the turnover were both carried out at 37 °C (Fig. 4C). Thus, we conclude that the accelerated pattern of turnover must have occurred immediately upon shifting the sec14c cki1 strain to 37 °C.

Phospholipid Composition—At 25 °C in I- medium, all four strains exhibited phospholipid compositions (Table III) similar to each other and to other published reports for these (12) and other strains (16) under similar growth conditions. The compositions of the sec14c cki1 and wild type strains grown in I- medium were also analyzed at 30 °C and were found to be similar to the compositions obtained at 25 °C. In I- medium at 37 °C, the three strains capable of growth (i.e. wild type, SEC14 cki1 and sec14c cki1) exhibited phospholipid compositions similar to those previously reported for this growth condition (12). In general, the phospholipid compositions of cells grown in I-, compared with I- medium, contained a higher proportion of PI (Table III). At 37 °C in I- medium, the sec14c cki1 strain, however, contained a proportion of PI (19% of total cellular phospholipids) approximately twice the proportion of PI observed in the wild type strain under these same growth conditions. With the exception of the sec14c cki1 strain at 37 °C, the PI content of all of the strains grown at 25 °C or 37 °C in I- medium was between 9–15% of the total phospholipid. In I- medium at 37 °C in all of the strains tested, the proportion of PI was 28–32% of total phospholipid (Table III).

Phospholipid Turnover—Cells were labeled to steady-state with 32P, as shown in Table III, and the retention and distribution of the 32P into the various phospholipids was tracked after shifting the cells into unlabeled medium (Fig. 6). At 25 °C, no distinctive differences among the four strains were observed (data not shown). At 37 °C, the patterns of 32P label retention in the wild type and SEC14 cki1 strains (Fig. 6, A and B) were similar to each other and to the results obtained in all four strains at 25 °C (data not shown). In all four strains at 25 °C, and in wild type SEC14 cki1 cells at 37 °C (Fig. 6, A and B), label was gradually lost from all lipids except PC, where it accumulated. In all cases except sec14c cki1 at 37 °C (Fig. 6C), the ratio of label remaining in PI versus PC tended to decrease over time (Fig. 6D). In sec14c cki1 at 37 °C, however, the PI/PC ratio started high and increased during the course of the 6-h turnover experiment. The label associated with the category “other,” which includes sphingolipids, also rose in the sec14c cki1 strain during the course of the turnover experiment at 37 °C. In the sec14c cki1 strain at 37 °C, sphingolipids accu-
mulated label during the course of the experiment (Fig. 6E). In the sec14\textsuperscript{es} chi1 strain, the proportion of label associated with PA remained fairly constant over time (Fig. 6C), whereas it dropped about 2-fold in the wild type (Fig. 6A) and in the SEC14 cki1 (Fig. 6B) strains in the first 3 h after transfer to unlabeled medium.

**Pulse Labeling of Phospholipids**—Because of the nature of the product-precursor relationships shown in Fig. 1, \textsuperscript{32}P introduced during a 30-min pulse labeling period will show a very different pattern of distribution than during steady-state labeling used to assess phospholipid composition (Table III). The three strains able to grow at 37 °C were pulse-labeled with \textsuperscript{32}P for 30 min, and in all three cases, the overall incorporation was greater per OD unit of culture in I\textsuperscript{1} medium compared with I\textsuperscript{2} medium (Table IV). The ratio of label recovered in association with PI compared with PC (PI/PC ratio) was higher in all three strains grown in I\textsuperscript{1} as opposed to I\textsuperscript{2} medium (Table IV). However, in I\textsuperscript{2} medium in the sec14ts cki1 strain, a higher proportion of label was associated with PI and there was a higher PI/PC ratio than in either the wild type strain or the SEC14 cki1 strain.

When examined at 25 °C, the sec14ts CKI1 strain showed a pattern of label incorporation during the 30-min pulse that was similar to the wild type strain at 37 °C. The sec14ts CKI1 and sec14ts cki1 strains were also pulse-labeled with \textsuperscript{32}P following a

### Table III

| Strain       | Medium | Temperature °C | PI (%) | PC (%) | PE (%) | PS (%) | PA (%) | Other (%) |
|--------------|--------|----------------|--------|--------|--------|--------|--------|-----------|
| wt (SEC14 CKI1) | I\textsuperscript{1} | 25              | 9.0    | 49.4   | 14.2   | 7.8    | 1.6    | 18.0      |
| sec14\textsuperscript{es} CKI1 | I\textsuperscript{1} | 30              | 11.9   | 48.2   | 13.3   | 5.6    | 1.3    | 19.8      |
| SEC14 chi1 | I\textsuperscript{1} | 30              | 12.7   | 47.4   | 15.9   | 5.0    | 0.9    | 18.1      |
| sec14\textsuperscript{es} chi1 | I\textsuperscript{1} | 30              | 12.9   | 44.5   | 15.9   | 5.9    | 1.5    | 19.3      |
| wt          | I\textsuperscript{2} | 30              | 10.8   | 46.8   | 13.4   | 8.4    | 1.6    | 19.0      |
| sec14\textsuperscript{es} CKI1 | I\textsuperscript{2} | 30              | 14.1   | 45.6   | 13.6   | 7.6    | 1.1    | 18.0      |
| wt          | I\textsuperscript{2} | 37              | 8.8    | 48.1   | 11.1   | 8.6    | 1.6    | 21.8      |
| SEC14 chi1 | I\textsuperscript{2} | 37              | 14.9   | 39.5   | 14.3   | 6.9    | 1.2    | 23.2      |
| sec14\textsuperscript{es} chi1 | I\textsuperscript{2} | 37              | 19.4   | 30.9   | 19.5   | 6.6    | 1.6    | 22.0      |
| wt          | I\textsuperscript{1} | 37              | 28.2   | 35.9   | 9.5    | 7.0    | 1.1    | 18.3      |
| SEC14 chi1 | I\textsuperscript{1} | 37              | 31.6   | 35.7   | 7.7    | 6.0    | 1.4    | 17.6      |
| sec14\textsuperscript{es} chi1 | I\textsuperscript{1} | 37              | 29.5   | 23.3   | 19.4   | 5.7    | 1.5    | 20.6      |

**Fig. 6. Phospholipid turnover as a function of growth temperature.** Strains were grown to mid-logarithmic phase in I\textsuperscript{1} medium supplemented with 10 µCi [\textsuperscript{32}P]orthophosphate/ml, as described in Table III. The starting phospholipid compositions were identical to those shown in Table III for each respective strain and growth conditions at 37 °C. The cells were harvested, washed, and inoculated into inositol-free non-radioactive media. At the indicated times, lipids were extracted and analyzed as described under “Experimental Procedures.”

The data shown in A, B, and C represent the relative percentages of the total label retained in each phospholipid species. “Other” lipids include CDP-DG, phosphatidylmonomethylethanolamine, phosphatidyltrimethylethanolamine, cardiolipin, and lipids retained near the origin. The ratio of PI/PC (D) was calculated from the relative percentage of \textsuperscript{32}P label associated with PI versus PC. Label associated with sphingolipid at 37 °C is depicted in E.
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TABLE IV

Pulse labeling of phospholipids

In A, strains grown to mid-log phase (A_{595} = 0.4–0.6) in I− or I+ medium at the indicated temperatures were pulsed for 30 min with 50 μCi/ml [32P]orthophosphate. The lipids were then extracted and analyzed as described under “Experimental Procedures.” In B, yeast cells were grown at 25 °C and then shifted to 37 °C. After 4 h of growth, the cells were pulse-labeled for 30 min with 50 μCi/ml [32P]orthophosphate. The lipids were extracted and analyzed. The amount of [32P] incorporated into lipid ([32P] Incorpor.) is presented as the counts/min per optical density unit at A_{595} × 10^3 (cpm/ODU × 10^3). The relative percentage of [32P] label in the individual phospholipid species is presented as a percentage of the total [32P] incorporated into lipid. The PI/PC ratio was calculated from the percentages of PI versus PC. “Other” lipids include CDP-DG, PMME, PDME, CL, as well as polar lipids that remained near the origin.

A. Pulse labeling at indicated temperatures

| Strain | Temperature | Medium | [32P] Incorpor. | [32P] in phospholipid (% of total) |
|--------|-------------|--------|----------------|-----------------------------------|
|        | °C          |        | cpm/ODU × 10^3 | PI       | PC       | PS       | PE       | PA       | Other | PI/PC |
| wt (SEC14 CKI1) | 37 | I+     | 213.4 | 42.3 | 6.7 | 22.4 | 11.4 | 2.5 | 14.7 | 14.7 | 6.31 |
| SEC14 chl1 | 37 | I+     | 175.4 | 45.2 | 5.8 | 21.3 | 11.8 | 2.7 | 13.2 | 7.79 |
| sec14ts chl1 | 37 | I+     | 167.8 | 39.9 | 6.4 | 15.3 | 26.9 | 2.0 | 9.5 | 6.23 |
| wt | 37 | I+     | 95.8  | 20.2 | 10.5 | 22.8 | 10.8 | 6.4 | 29.2 | 19.2 |
| SEC14 chl1 | 37 | I+     | 101.4 | 24.1 | 6.6 | 24.3 | 11.6 | 6.0 | 27.5 | 3.65 |
| sec14ts chl1 | 37 | I+     | 93.8  | 30.0 | 6.4 | 20.4 | 27.8 | 3.0 | 12.4 | 4.89 |
| sec14ts CKI1 | 25 | I+     | 163.2 | 37.5 | 6.2 | 24.6 | 17.1 | 2.3 | 12.2 | 6.05 |
| sec14ts CKI1 | 25 | I+     | 130.6 | 17.3 | 15.0 | 25.1 | 12.6 | 6.5 | 23.6 | 1.15 |

B. Pulse labeling after temperature shift from 25 °C to 37 °C

| Strain | Medium | [32P] Incorpor. | [32P] in phospholipid (% of total) |
|--------|--------|----------------|-----------------------------------|
|        |        | cpm/ODU × 10^3 | PI       | PC       | PS       | PE       | PA       | Other | PI/PC |
| sec14ts CKI1 | | 36.6  | 41.7 | 23.8 | 12.0 | 8.4 | 2.7 | 11.4 | 1.75 |
| sec14ts CKI1 | | 16.4  | 21.8 | 27.9 | 16.3 | 6.9 | 5.1 | 22.0 | 0.78 |
| sec14ts chl1 | | 277.2 | 41.1 | 7.9 | 14.9 | 24.6 | 2.8 | 8.7 | 5.20 |
| sec14ts chl1 | | 74.0  | 30.3 | 8.4 | 19.3 | 19.4 | 4.6 | 18.0 | 3.61 |

Fig. 7. Northern blot analysis of INO1 expression in wild type, SEC14 chl1, and sec14ts chl1 strains at 25 °C and 37 °C. Strains were grown in I− or I+ media, as indicated. RNA was extracted and Northern blot analysis was performed as described under “Experimental Procedures.” Hybridization with the TCM1 probe served as a loading control.

INO1 lacZ reporter construct, described under “Experimental Procedures.” This construct was transformed into the four strains used to study INO1 regulation, and the strains were assayed for β-galactosidase activity under various growth conditions. To establish that the reporter construct was regulated in a fashion resembling the native INO1 transcript (Fig. 7; Table V), we initially studied expression of β-galactosidase in cells grown to mid-logarithmic phase (Table VI). The sec14ts CKI1 strain will not grow at 37 °C and, therefore, only three of the four strains tested under any growth condition (Table IV, part B).

Regulation of the INO1 Gene in the sec14ts chl1 Strain—The Opi− phenotype, observed in the sec14ts strain at 37 °C, is associated with misregulation of INO1 (19). Therefore, we investigated INO1 regulation in four strains: wild type (SEC14 CKI1), sec14ts CKI1, SEC14 chl1, and sec14ts chl1. At 25 °C, all four strains showed repression of the INO1 transcript in response to inositol when tested by Northern blot analysis (Fig. 7; Table V). The sec14ts CKI1 strain was also examined at 30 °C and showed normal regulation. At 25 °C or 30 °C in each of these strains, the INO1 transcript was substantially repressed (i.e. 10-fold or more) in cells grown in I+ medium compared with cells grown in I− medium (Fig. 7; Table V).

At 37 °C, only three of the strains will grow: wild type, SEC14 chl1, and sec14ts chl1; the wild type and SEC14 chl1 strains both exhibited INO1 normal regulation at 37 °C (Table V, Fig. 7). However, consistent with its Opi− phenotype, the sec14ts chl1 strain showed an abnormal pattern of INO1 expression at 37 °C. In the absence of inositol, the INO1 transcript was expressed at a level somewhat higher than the wild type derepressed level. Furthermore, when inositol was added to the growth medium, in the absence of choline, the level of INO1 transcript was repressed only about two-fold, compared with more than 10-fold repression seen in the wild type strains (Table V). When inositol and choline were added together to the growth medium, no repression of INO1, at all, was seen in the sec14ts chl1 strain at 37 °C (Table V).

Kinetics of INO1 Induction in the sec14ts chl1 Strain Shifted to 37 °C—To study the kinetics of INO1 induction, we used the shift from 25 °C to 37 °C in I− or I+ media. The labeling pattern of the sec14ts chl1 strain under these conditions (Table IV, part B) was quite similar to the pattern seen when the same strain was maintained continuously at 37 °C in I− or I+ media. However, the sec14ts CKI1 strain incorporated less [32P] label per OD unit of culture 4 h after the shift to 37 °C as compared with the sec14ts chl1 strain, and a much higher proportion of the label was incorporated into PC in the sec14ts CKI1 strain at 37 °C than in any other strain tested under any growth condition (Table IV, part B).

INO1 lacZ reporter construct, described under “Experimental Procedures.” This construct was transformed into the four strains used to study INO1 regulation, and the strains were assayed for β-galactosidase activity under various growth conditions. To establish that the reporter construct was regulated in a fashion resembling the native INO1 transcript (Fig. 7; Table V), we initially studied expression of β-galactosidase in cells grown to mid-logarithmic phase (Table VI). The sec14ts CKI1 strain will not grow at 37 °C and, therefore, only three of the four strains, namely wild type, SEC14 chl1, and sec14ts chl1, were tested at 37 °C. The overall pattern of expression of the INO1 lacZ construct (Table VI) was very similar to the expression of the native INO1 transcript measured by Northern blot analysis (Table V; Fig. 7). At 25 °C and 30 °C, all four strains showed β-galactosidase regulation in response to inositol comparable to wild type, except that the sec14ts chl1 strain had a slightly higher level of β-galactosidase under repressing conditions (I− medium). At 37 °C, the wild type and SEC14 chl1 strains, showed the normal pattern of regulation, i.e. repression of INO1 lacZ in response to inositol, while the sec14ts chl1 strain exhibited misregulation of INO1 lacZ at 37 °C (Table
Effect of inositol and choline on INO1 gene expression as measured by Northern blot quantitation

Table gives quantitation of Northern blot analysis shown in Fig. 4. Strains were grown in I- or I+ medium with (C+) or without (C-) 1 mM choline. RNA analysis was performed as described under “Experimental Procedures.” Hybridization with the TCM1 probe served as a loading control. Quantitation was performed with an AMBIOS 4000 phosphorimager. Data are expressed as a percentage of the expression observed in the wild type (wt) strain at 37 °C in I- conditions.

| Strain          | Temperature | INO1 expression (%) |
|-----------------|-------------|---------------------|
|                 | C-         | C+      | C-      | C+      |
| wt (SEC14 CKI1) | 25         | 116     | NP*     | 6       | NP      |
| sec14 CKI1      | 25         | 83      | 57      | 6       | 6       |
| SEC14 chl1      | 25         | 153     | NP      | NP      | 16      |
| sec14 chl1      | 25         | 103     | 119     | 6       | 9       |
| sec14 CKI1      | 25         | 150     | 116     | NP      | NP      |
| wt              | 37         | 100     | NP      | NP      | 13      |
| SEC14 chl1      | 37         | 128     | NP      | NP      | 19      |
| sec14 chl1      | 37         | 163     | 163     | 72      | 209     |

* NP, not performed.

Effect of inositol and temperature on INO1 gene expression as measured by β-galactosidase activity

Various yeast strains were transformed with plasmid pH359 as described under “Experimental Procedures.” The strains were grown in I- or I+ medium under comparable conditions. The bulk of the [14C]choline excreted by the wild type strain grown under comparable conditions (Fig. 8, compare F (sec14 CKI1) and H (wild type)). When shifted from 25 °C to 37 °C in I- medium, no derepression of the INO1 construct continued after the sec14 chl1 cells became stationary. The sec14 CKI1 strain stopped growing after 5–6 h upon shifting to 37 °C (Fig. 8E).

DISCUSSION

Studies using wild type yeast cells have detected very little PC turnover (32). Consistent with these earlier studies, our analysis of PC turnover in wild type yeast using both 32P label and [14C]choline label, revealed very little evidence of extensive PC turnover (Figs. 4–6). In cells carrying a mutation in the CDP-choline pathway, it is possible to examine phospholipid metabolism under conditions in which reutilization of the choline liberated by PC turnover is largely blocked. Under these circumstances, we detected substantial PC turnover and the choline liberated by this process appeared in the growth medium, only after the initial burst of choline liberation (10–12). Our analysis substantiates this hypothesis.

The cholineretention experiment provides a means of estimating the extent of activity in the CDP-choline pathway in cells growing in the absence of choline. Recent studies have suggested that this pathway contributes substantially to PC biosynthesis even in the absence of exogenous choline (10–12). Our analysis substantiates this hypothesis. Reutilization of choline liberated by turnover is, apparently, a major function of the CDP-choline pathway in yeast cells growing in the absence of choline. Based on the choline excreted by the SEC14 chl1 strain at 37 °C (Figs. 4 and 5), we estimate that some 7% of cellular choline is recycled via PC turnover and re-synthesis in each generation period (about 3 h). This must be an underestimate because chl1 cells retain some capacity to reuse free choline, as evidenced by our ability to label the cells with [14C]choline in the first place. Nevertheless, it is possible to use the choline excretion plate phenotype (Opc-) to carry out a qualitative comparison of the extent of PC turnover in different strains, under different growth conditions. For example, in the SEC14 chl1 strain, we observed that the extent of the choline excretion ring was affected by temperature and by the presence of inositol (Fig. 5, Table II).

The Nature of PC Turnover in Yeast—We propose that the free choline found in the growth medium of the chl1 bearing strains most likely arises from phospholipase D (PLD)-mediated PC turnover which produces free choline and phosphatidic acid (PA). It is now recognized that PLD-mediated hydrolysis is a major route of PC breakdown in a variety of mammalian cell types (36). The bulk of the [14C]choline excreted by the SEC14 chl1 strain detected in the growth medium was free choline (Fig. 5). This is not consistent with phospholipase B (PLB)-mediated turnover, which produces glycerophosphocholine.
FIG. 8. Kinetics of INO1 derepression in sec14 and sec14\textsuperscript{+} cki1 mutants. Yeast strains containing plasmid pH359 were grown at 25 °C under repressing conditions (I\textsuperscript{−} medium), to the mid-logarithmic phase of growth. The cells were harvested by filtration and transferred to fresh media (I\textsuperscript{−} or I\textsuperscript{+}) at the permissive (25 °C) or non-permissive (37 °C) temperature. At the indicated time points, the A\textsubscript{595} of the cultures (O) and \(\beta\)-galactosidase activity (\(\Delta\)) was determined. Graphs A–D represent the sec14\textsuperscript{+} cki1 strain shifted to: I\textsuperscript{−} medium at 25 °C (A), I\textsuperscript{+} medium at 25 °C (B); I\textsuperscript{−} medium at 37 °C (C); and I\textsuperscript{+} medium at 37 °C (D). Graphs E and F represent the sec14\textsuperscript{−} CKI1 strain shifted to I\textsuperscript{−} medium at 37 °C (E) and I\textsuperscript{−} medium at 37 °C (F). Graphs G and H represent the wild type (SEC14 CKI1) strain shifted to I\textsuperscript{−} medium at 37 °C (G) and I\textsuperscript{−} medium at 37 °C (H).
suggest that the bulk of PC turnover in yeast cells is carried out in a lipid-associated pool (3) that is cycled into water-soluble products such as choline phosphoethanolamine (PE) and is then recycled as phosphatidylcholine (PC) (15, 46, 47). Similar to mammalian cell types, yeast contain high PLD activity (39). Ktistakis et al. (39) showed that PA produced by PLD-mediated turnover was sufficient for coatomer binding to Golgi membranes even in the absence of ADP-ribosylation factor. PLD-mediated turnover also hydrolyzes PC, high levels of which may interfere with vesicle formation and/or membrane fusion events required for the ongoing secretory pathway. In the sec14ts cki1 strain, elevation to the restrictive temperature leads to both increased turnover of PC and to increased net PI synthesis. The combination of these effects leads to the restoration of the PI/PC balance, which McGee et al. (12) proposed is important in the ongoing secretory pathway. We propose that the increased synthesis of PI is due both to the greater availability of the PA precursor and also to the regulatory link to INO1 regulation, which we discuss below. Increased PI biosynthesis could lead, in turn, to increased synthesis of PIP and PIP_2, all of which are negatively charged phospholipids, expected to facilitate the ongoing secretory process. PIP and PIP_2 are also known to stimulate PLD activity in yeast as in mammals (40). This argument is consistent with the fact that Sec14p dysfunction results in increased PC content in Golgi membranes (12) and that Golgi enriched membranes from mammalian cell types have high PLD activity (39).

Role of PC Turnover in Regulation of the INO1 Gene—We propose that the derepression of the INO1 gene, which occurs rapidly following the shift of the sec14ts cki1 strain to 37 °C (Fig. 8) even in the presence of inositol, is directly correlated to and caused by the increase in the rate of PC turnover that occurs when Sec14p is inactivated. It has long been known (16, 41) that there is an association between PC and inositol metabolism. Structural gene mutants with defects at every step (Fig. 1) in the PA to PC pathway (i.e. \( \text{PA} \rightarrow \text{CDP-DG} \rightarrow \text{PS} \rightarrow \text{PE} \rightarrow \rightarrow \rightarrow \rightarrow \text{PC} \)) exhibit Opi− phenotypes and misregulate INO1 and other co-regulated genes (for reviews, see Refs. 16, 42, and 43). The structural gene mutants in this series include the \( cdg1(cds1) \) mutant (44), which is defective in CDP-DG synthase (45), which catalyzes the first step in this reaction series (i.e. \( \text{PA} \rightarrow \text{CDP-DG} \) and the opl3 mutant, which is defective in PI methyltransferase, which catalyzes the last two reactions (i.e. phosphatidylmonomethylethanolamine \( \rightarrow \) phosphatidylcholine (PC) (15, 46, 47). Similar Opi− phenotypes are present in structural gene mutants defective in enzymes for every other metabolic step in the sequence.
between PA and PC (Fig. 1), including cho1, (48) cho2, (9, 13), and \( \text{psd}1^{+} \).

In the current study, we demonstrate that elevation of the \( \text{sec}1^{4} \) \( \text{cki}1 \) strain to the restrictive temperature results in increased turnover of PC and also generates a signal for derepression of \( \text{INO}1 \). The derepression of \( \text{INO}1 \) that we observed upon shift to the restrictive temperature exhibits remarkably rapid kinetics (Fig. 8) and correlates to the increase in PC turnover. In a previous study (9), we employed strains containing combinations of mutations in the PE methylation and CDP-choline routes for PC biosynthesis. In that study, we demonstrated that no single metabolite in either pathway for PC biosynthesis correlated to the production of the signal for repression/derepression of \( \text{INO}1 \). The metabolic conditions previously tested for their roles in \( \text{INO}1 \) repression/derepression included relative PC content and free choline availability. Neither of these factors was correlated to \( \text{INO}1 \) derepression (9). Rather, the metabolic signal controlling \( \text{INO}1 \) regulation appeared to be linked to the overall ability of increased PC biosynthesis to stimulate cell growth (9). The data presented in the current study suggests that \( \text{INO}1 \) derepression is correlated to PC turnover. We propose that \( \text{INO}1 \) derepression occurs in response to a metabolic signal generated in the course of the overall alteration in phospholipid metabolism produced by increased PC turnover. We are currently testing these ideas in cells containing mutations in \( \text{PLD}1 \), the major phospholipase D of the yeast cell (49–51), first isolated as \( \text{SPO14} \) (35, 40).

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4 P. Griac, unpublished data.
Role of the Yeast Phosphatidylinositol/Phosphatidylcholine Transfer Protein (Sec14p) in Phosphatidylcholine Turnover and INO1 Regulation
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