Nte1p-mediated Decaylation of Phosphatidylcholine Functionally Interacts with Sec14p*

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Deciphering the function of the essential yeast Sec14p protein has revealed a regulatory interface between cargo secretion from Golgi and lipid homeostasis. Abrogation of the CDP-choline (CDP-Cho) pathway for phosphatidylcholine (PC) synthesis allows for life in the absence of the otherwise essential Sec14p. Nte1p, the product of open reading frame YML059c, is an integral membrane phospholipase against CDP-Cho-derived PC producing intracellular glycerophosphocholine (GP Cho) and free fatty acids. We monitored Nte1p activity through in vivo PC turnover measurements and observed that intracellular GPCcho accumulation is decreased in a sec14ts strain shifted to 37 °C in 10 mM choline (Cho)–containing medium compared with a Sec14p-proficient strain. Overexpression of two Sec14p homologs Sfh2p and Sfh4p in sec14ts cells restored secretion and growth at the restrictive temperature but did not restore GPCcho accumulation. Instead, newly synthesized PC was degraded by phospholipase D (Spo14p). Similar analysis performed in a sec14A background confirmed these observations. These results imply that the ability of Sfh2p and Sfh4p to restore secretion and growth is not through a shared function with Sec14p in the regulation of PC turnover via Nte1p. Furthermore, our analyses revealed a profound alteration of PC metabolism triggered by the absence of Sec14p: Nte1p unresponsiveness, Spo14p activation, and deregulation of Pct1p, Sfh2p– and Sfh4p-overexpressing cells coped with the absence of Sec14p by controlling the rate of phosphocholine formation, limiting the amount of Cho available for this reaction, and actively excreting Cho from the cell. Increased Sfh4p also significantly reduced the uptake of exogenous Cho. Beyond the new PC metabolic control features we ascribe to Sfh2p and Sfh4p, we describe a second role for Sec14p in mediating PC homeostasis. Sec14p acts as a positive regulator of Nte1p-mediated PC deacylation with the functional consequence of increased Nte1p activity increasing the permissive temperature for the growth of sec14ts cells.

Lipid homeostasis is fulfilled through the coordinated synthesis, degradation, and trafficking of the lipid constituents of biological membranes. Many biological processes such as vesicle formation, protein trafficking, and lipid signaling are dependent on proper lipid content at particular membrane locations, and many cellular pathophysiological situations are associated with perturbations in lipid homeostasis (1).

Deciphering the function of the Saccharomyces cerevisiae Sec14p protein has uncovered a regulatory interface between cargo secretion from the Golgi and lipid metabolism (2, 3). Sec14p is an essential soluble protein possessing in vitro phosphatidylinositol (PI)²/²phosphatidylcholine (PC) transfer activity. Sec14p is located primarily in the cytoplasm, and it has been observed to associate with Golgi membranes presumably through its phospholipid binding ability (4). The lethality associated with Sec14p dysfunction or absence can be overcome by inactivating mutations in each of the three structural genes of the CDP-choline (CDP-Cho) pathway for PC biosynthesis (4). In yeast PC can also be synthesized through the methylation pathway where phosphatidylethanolamine is methylated sequentially yielding PC (5, 6). Abrogation of the CDP-Cho pathway functionally complements the absence of Sec14p (2, 4, 5, 7). It was also shown that Sec14p bound to PC inhibits by an unknown mechanism CTP:phosphocholine cytidylyltransferase (Pct1p) activity (7), the rate-determining enzyme of the CDP-Cho pathway (8, 9). Consistently, Sec14p overexpression effects a measurable reduction in flux through this pathway (7). Two other genes that when inactivated allow cell growth and secretion on the absence of Sec14p are SAC1 and KES1. Both genes are also involved in lipid metabolism and support the notion that the prosecretory function of Sec14p interfaces with lipid homeostasis (10–12).

SFH2 and SFH4 belong to the Sec 14 homolog gene family (SFH1–5). Both SFH2 and SFH4 protein products exhibit in vitro PI transfer activity but do not possess the PC transfer activity of Sec14p. Despite their inability to bind PC, the overexpression of Sfh2p and Sfh4p, but not other members of the Sfh family, fully restores growth in the absence of Sec14p (13–15).

Type B and D phospholipases have been described in yeast with activity against PC. Phospholipase D breaks a phosphoester bond producing phosphatidic acid and choline (Cho). The sole PC phospholipase D in yeast is Spo14p, a soluble protein primarily found in the cytoplasm but with the capacity to translocate to cellular membranes for substrate degradation (16–19). Spo14p has an essential function for sporulation but is dispensable for vegetative growth. Interestingly, inactivation of Sec14p function promotes Spo14p activity, and the ability of all the genes whose inactivation can bypass the essential function of Sec14p is fully dependent on functional Spo14p (20–22).

The abbreviations used are: PI, phosphatidylinositol; CDP-Cho, CDP-choline; Cho, choline; GPCho, glycerophosphocholine; NTE or Nte, neuropathy target esterase; ORF, open reading frame; PC, phosphatidylcholine; P-Cho, phosphocholine; Pct1p, CTP:phosphocholine cytidylyltransferase.

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Phospholipase B deacylates PC-producing glycerophosphocholine (GPCho) and free fatty acids. Three different genes coding for phospholipase B activities (PLB1-3) have been identified in S. cerevisiae whose protein products are located at the plasma membrane and within the periplasmic space (23, 24). Plb1p supplies the main activity responsible for PC deacylation at the plasma membrane with its production of GPCho released into the extracellular medium. Dowd et al. (25) reported a PC deacylating activity responsible for the production of intracellular GPCho. This activity was induced by the presence of Cho in the culture medium or when the growth temperature was raised to 37 °C. It was shown that the stimulating effects of the addition of Cho and of the elevation of temperature on the deacylating activity were dependent on an active CDP-Cho pathway for PC biosynthesis. The yeast open reading frame (ORF) YML059c was recently found to encode for this activity, and the predicted protein sequence has high similarity to the human neuropathy target esterase protein (26). Both the human and yeast proteins are PC-deacylating serine hydrolases, located in the endoplasmic reticulum, and exhibit similar sensitivities against organophosphorus esters.

After a decade of intense investigation a precise molecular depiction of the role played by Sec14p in protein secretion and cell viability remains elusive, but cumulative evidence supports a view whereby Sec14p keeps the lipid composition of internal cellular compartments competent for cargo trafficking. We present metabolic and genetic evidence showing that internal cellular compartments competent for cargo trafficking.

**EXPERIMENTAL PROCEDURES**

Materials—Choline oxidase (from Arthrobacter globiformis) and horseradish peroxidase were from Sigma. Radiolabeled [methyl-14C]Cho was purchased from American Radiolabeled Chemicals. Silica gel thin layer chromatography plates were purchased from Whatman.

**Yeast Strains, Culture Conditions, and Plasmid Construction—**

Standard molecular biology methods, yeast genetic techniques, and gel thin layer chromatography plates were purchased from Whatman.

**Choline Transport Assays—**

The transport of choline was determined as described. Total uptake was taken to be the radioactivity incorporated into the cells at zero time point of the chase.

**Choline Transport Assays—**

Cells were grown to mid log phase in minimal glucose medium containing the nutritional requirements for cell growth at 25 °C, and choline transporter activity was determined essentially as described with the following minor modifications. Cells were harvested, washed with fresh medium, and resuspended in identical medium containing 1 μM [14C]Cho for 30 min at 25 °C. Yeasts were centrifuged, washed in fresh medium, and recultured in identical medium containing 10 μM nonradiolabeled Cho prewarmed at 25 or 37 °C. Aliquots were removed at various time points, and cells were pelleted by centrifugation. The supernatant (medium fraction) and the cells were submitted to radiolabel metabolism analysis and lipid phosphorous determination as described. Total uptake was considered the radioactivity incorporated into the cells by guest on July 18, 2018http://www.jbc.org/Downloaded from
Whatman GF/C glass microfiber filters and rinsed twice with 25 ml of ice-cold phosphate-buffered saline containing 1 mM Cho. The filters were allowed to dry, and the associated radiolabel was determined by liquid scintillation counting.

**PC Biosynthesis**—Mid-log phase yeast grown at 25 °C were centrifuged at 2,200 × g for 5 min, washed in fresh medium, and recultured in identical medium containing 10 μCi (4,000 dpm/nmol) [14C]Cho at 25 °C (6, 8, 9, 18). Aliquots were withdrawn at the indicated time points for radiolabeled metabolite analysis and lipid phosphorus determination.

**Analysis of Radiolabeled Cho-containing Metabolite**—Yeast cells were centrifuged at 2,200 × g for 5 min at 4 °C. Cells were washed twice with ice-cold water, resuspended in 1 ml of CHCl3 and CH3OH (1/1, v/v), and disrupted for 1 min at 4 °C using a BioSpec Multi-bead Beater containing 0.5 g of 0.5-mm acid-washed glass beads. The beads were washed with 1 ml of CHCl3 and CH3OH (2/1, v/v), and 1.5 ml of water and 1 ml of CHCl3 and CH3OH (5/1, v/v) were added to the combined supernatant to facilitate phase separation. Phospholipids in the organic phase were analyzed by thin layer chromatography on Whatman Silica Gel 60A plates using the solvent system CHCl3, CH3OH, H2O, CH3COOH (70/30/2/2, v/v/v/v). Cho-containing metabolites in the aqueous phase were separated in a solvent system consisting of CH3OH, 0.6% NaCl, NH4OH (50/50/5, v/v/v) (6, 8, 9, 18). Plates were scanned with a BioSkan radionuclear imaging scanner, and the corresponding bands were scraped into vials for liquid scintillation counting. Metabolite identity was based on the mobility of known standards dissolved in unlabeled aqueous intracellular fraction or unlabeled cultured medium.

**Cho Content Measurement**—Yeast cells growing logarithmically in 200 ml of minimal supplemented glucose medium at 25 °C were cooled rapidly in ice water, harvested, and washed with a volume of ice-cold 5 mM NADP, 5 mM KF, and 0.1 mM phenylmethylsulfonyl fluoride. The metabolic poisons were removed by three additional ice-cold water washes. Aliquots of cells (100 mg) were transferred into a 1.5-ml tube containing 0.8 g of acid-washed glass beads. One ml of CHCl3 and CH3OH (1/1, v/v) cooled at −20 °C and 50 μl of 40,000 dpm [14C]Cho (55 μCi/nmol) were added. Cells were immediately broken by vigorous agitation using a bead beater (four bursts of 90 s with cooling on an ice-aisy mix between bursts). After storage overnight at −20 °C the extract was transferred to a new tube, and the beads were washed twice with 0.5 ml of CHCl3 and CH3OH (2/1, v/v). To facilitate phase separation 1 ml of CHCl3 and CH3OH (5/1, v/v) and 1.5 ml of water were added. The tubes were agitated for 10 min at 250 rpm and centrifuged at 2,200 × g for 10 min. Two ml of the aqueous phase was removed and concentrated to 0.5 ml. A white interface containing cellular debris was aspirated, and an aliquot of organic phase was saved for lipid phosphorus determination. The entire aqueous phase was chromatographed through a column (2.7 × 18 mm) of Dowex AG 1-X8 (OH form) developed with water (8). Cho eluting in the flow-through was washed from the column, concentrated to 0.55 ml, and clarified by centrifugation at 18,000 × g for 10 min. The mass of Cho contained in 0.5 ml of supernatant was estimated using the choline oxidase-peroxidase coupled enzyme assay (28). Recovery of Cho from each cellular extract was estimated based on the yield of [14C]Cho contained in the supernatant.

**Standard Methods**—Protein mass was determined using the Lowry method (29) and lipid phosphorus as described by Ames and Dubin (30).

**RESULTS**

**Intracellular GPCho Accumulation Rate Is Responsive to Sec14p**—The product of the yeast YML059c ORF is homologous to mammalian neuropathy target esterase and is responsible for deacylating PC synthesized through the CDP-Cho pathway producing intracellular GPCho and free fatty acids (25, 26).

Because the identification of the gene coding for this activity was based on its similarity with the human neuropathy target esterase gene we have proposed NTE1 and Nte1p as the name for the YML059c ORF and its protein product, respectively. This activity was shown to increase concurrently with augmentation of the synthesis of PC induced by either the addition of exogenous Cho or elevation of temperature (25, 26). Using a nte1Δ::kanMX null strain and its corresponding wild type we performed pulse-chase analysis of PC turnover. Yeast were labeled with [14C]Cho for 30 min at 25 °C, and the label was chased after the addition of 10 mM nonradioactive Cho at 25 or 37 °C (Fig. 1). The time course analysis of label distribution among Cho-containing metabolites from aqueous and organic intracellular fractions as well as extracellular medium during the chase in both strains revealed that the nte1Δ mutant strain did not produce intracellular GPCho after the addition of 10 mM Cho at either 25 or 37 °C. The wild type strain exhibited a basal amount of intracellular GPCho production at the end of the pulse period which increased slightly during the chase period at 25 °C and dramatically during the chase at 37 °C. This observation is consistent with previous reports indicating that Nte1p is the main activity, if it is not only, responsible for deacylating PC synthesized through the CDP-Cho pathway that leads to an accumulation of intracellular GPCho (25, 26).

The amount of radiolabeled Cho excreted from the nte1 null strain was ~3-fold higher than the amount in the medium of the wild type strain when the chase was performed at 37 °C.
existed we performed \(^{14}\)C Cho pulse-chase experiments to
investigate whether relevant features to control the abundance of PC made through the CDP-Cho pathway located in the endoplasmic reticulum, Nte1p seemed to meet all criteria demanded of phospholipase against CDP-Cho-derived PC that is involved in the formation of extracellular metabolites during a 90-min chase period performed at 25 °C. At time zero, cells were harvested, washed, and recultured in identically supplemented medium containing 10 mM nonradioactive Cho warmed at 37 °C. At the indicated times, aliquots of the culture were analyzed for radioactivity distributed among the indicated Cho-containing metabolites. Total radioactivity incorporated into the five strains was similar (32.4 ± 3.1, 36 ± 1.1, 36.4 ± 1.6, 36.1 ± 2.6, and 36.9 ± 1.0 nCi/10^7 cells for pRS413-SEC14, YEp-HIS3-SEC14^K66,239A, vector control, YEp-URA3-SFH2, and YEp-URA3-SFH4-transformed sec14ts strains, respectively). Data are expressed as a percentage of total label recovered in each fraction at each time point after the shift to unlabeled medium. This experiment was repeated four times with qualitatively similar results.

With respect to total PC turnover, this increased amount of excreted Cho did not compensate for the absence of intracellular GPCho production in the nte1 null strain because 60% of the label was associated with PC after 90 min of chase in the nte1 null strain compared with only 20% of the label remaining with PC in the wild type strain at the same time point. Total Cho uptake during the pulse-chase experiment (Fig. 1) as well as direct Cho transporter measurements (9) performed under linear time conditions (data not shown) indicated that the nte1Δ strain took up Cho at the same rate as the wild type strain during the pulse phase at 25 °C.

Based on the compelling evidence for a role for Sec14p in regulating the CDP-Cho pathway for PC biosynthesis to maintain secretory proficiency from Golgi membranes (4, 31, 32) we hypothesized that Sec14p might play in this scenario. As a devoted phospholipase against CDP-Cho-derived PC that is located in the endoplasmic reticulum, Nte1p seemed to meet relevant features to control the abundance of PC made through the CDP-Cho pathway at that location. To investigate whether a possible functional interaction between Nte1p and Sec14p existed we performed \(^{14}\)C Cho pulse-chase experiments to measure the rate of PC turnover in the CTY1-1A (sec14ts) strain transformed with a low copy plasmid bearing the SEC14 wild type allele, a low copy vector bearing the SEC14^K66,239A allele (33), and empty vector. Wild type Sec14p possesses PC transfer activity, whereas Sec14^K66,239A p possesses PC transfer activity, but it is unable to transfer PI. Currently, relevant phenotypic differences between Sec14^K66,239A p allele and wild type Sec14p in yeast have not been reported.

The variation of distribution of the label among the Cho-containing metabolites during a 90-min chase period performed with 10 mM nonradioactive Cho at 37 °C in the sec14ts background is shown (Fig. 2). Under these chase conditions (10 mM Cho at 37 °C) Sec14p is inactivated, and conversely Nte1p is fully activated. At the first two chase time points analyzed (0 and 15 min) the metabolic profile of sec14ts cells is similar to those of SEC14- and SEC14^K66,239A-containing strains. A steady increase in the formation of intracellular GPCho was observed in the SEC14-containing strain at longer chase periods (up to 90 min). The SEC14^K66,239A-containing strain exhibited a sustained rate of intracellular GPCho formation during the chase period, but it was less pronounced than that observed in SEC14 cells. In contrast, in the sec14ts strain the percentage of label associated with intracellular GPCho at the 15-min chase remained almost unchanged as the chase period was prolonged. It should be noted that at 37 °C the CTY1-1A strain is no longer viable, in contrast with the SEC14 and SEC14^K66,239A strains, which grow at that temperature.

Overexpression of Sh2p and Sh4p allows yeast to grow in the otherwise lethal absence of a functional Sec14p (13). The mechanism for rescuing the absence of Sec14p by overexpression of Sh4p is dependent on the presence of Spo14p, whereas Spo14p slightly improves the rescue of sec14-associated defects via overexpression of Sh2p (13). We performed pulse-chase analysis of PC turnover in the CTY1-1A (sec14ts) strain expressing Sh2p or Sh4p from high copy plasmids. Surprisingly, radioactivity distribution profiles corresponding to Sh2p- and Sh4p-overexpressing strains were strikingly similar to that corresponding to the sec14ts strain (Fig. 2). The percentage of radioactivity associated with intracellular GPCho did not rise after an initial increase during the 15-min chase point for both the SFH2- and SFH4-containing strains. These results suggest that reduced intracellular GPCho accumulation is related to the absence of a functional Sec14p and not to the lethality emerging from its deficiency. Furthermore, these results imply that the ability of Sh2p and Sh4p to restore secretion and growth is not through a shared function with Sec14p in the regulation of PC turnover. The percentage of label associated with extracellular metabolites slightly increased for the sec14ts strain alone and for the sec14ts strains overexpressing SFH2 or SFH4. It is known that inactivation of Sec14p evokes stimulation of Spo14p. Furthermore, in all known “Sec14 bypass” mechanisms Spo14p activity is an essential player to overcome the absence of a functional Sec14p, with the exception of overexpression of Sh2p-mediated rescue (13). It is interesting to note a higher extent of radiolabeled Cho excretion in SFH4-overexpressing cells compared with SFH2 cells (and with sec14ts strain). The different profiles of Cho excretion between these strains likely reflect their dissimilar Spo14p dependence.
Sec14p Regulation of PC Deacylation

Because all of the strains analyzed incorporated a similar amount of radiolabeled Cho/cells during the 30-min pulse period (performed at 25 °C) the changes in the percentage of total radioactivity associated with each Cho-containing compound during the chase period reflect quantitative differences in rates of Cho-containing metabolite alterations.

To understand further the role of Sec14p and its homologs SFH2p and SFH4p on the regulation of PC turnover we performed pulse-chase experiments under conditions where the temperature shift to 37 °C was no longer necessary to induce loss of function of Sec14p via use of a sec14ts allele. Four different sec14Δ haploid strains bearing SEC14 or SEC14K66,239A on low copy plasmids, and SFH2 or SFH4 on high copy plasmids were obtained by sporulation of corresponding transformed sec14Δ::kanMX diploids. All four sec14Δ strains were viable at both 25 and 37 °C when containing SEC14 or SEC14K66,239A on low copy plasmids or SFH2 or SFH4 on high copy plasmids and exhibited similar growth rates in defined medium (Table III). We first analyzed the fate of [14C]Cho-containing metabolites during a 90-min chase period performed with 10 mM unlabeled Cho at 25 °C (see Fig. 3A). This augmented intracellular GPCCho production upon temperature elevation is a signature of Nte1p activity (25, 26). However, SEC14- and SEC14K66,239A-containing strains exhibited a sustained rate of GPCCho accumulation during the entire chase period, whereas SFH2 and SFH4 cells did not. After an initial increase in intracellular GPCCho label detected up to the 15-min chase the label associated with this metabolite remained almost unchanged up to end of the chase in the SFH2- and SFH4-containing strains. In fact, the percentage of label associated with intracellular GPCCho in SFH4 cells decreased slightly at 60 and 90 min. In an overall comparison, the intracellular GPCCho accumulation profiles observed in the sec14Δ background at 37 °C (Fig. 3B) resemble those recorded in the sec14Δ background at the same temperature (Fig. 2). The profiles of Cho and GPCCho excretion in the four strains analyzed at 37 °C did not differ significantly from those observed when the chase was performed at 25 °C (Fig. 3, A and B).

PC Metabolism in sec14Δ Cells—Another fact must be highlighted regarding the effects of the absence of Sec14p on PC metabolism: the total label taken up by Sfh2p- and Sfh4p-overexpressing cells during the pulse period at 25 °C was reduced about 60–70% compared with the label taken up by SEC14 and SEC14K66,239A strains (see legend of Fig. 3). This decrease in label uptake was not observed when the [14C]Cho pulse was performed in a sec14Δ background at the permissive temperature of 25 °C (see legend of Fig. 2).

To determine whether the detected diminutions in Cho uptake in Sfh2p- and Sfh4p-overexpressing cells in a sec14Δ background were the result of changes in the activity of the Cho transporter short pulse (2 min and 4 min) Cho uptake measurements were performed (9). The rate of Cho uptake was linear during this interval for all strains analyzed. The SEC14 and SEC14K66,239A strains exhibited almost identical velocities of Cho uptake (Table III). SFH2 cells the activity of the transporter was only slightly reduced, whereas in contrast choline transporter activity was reduced to 25% in SFH4 cells. Because overexpression of Sfh2p and Sfh4p in a Sec14p-proficient background had no measurable effect on the amount of Cho taken up during the pulse period (Fig. 2), and the profiles of radioactivity distribution among the different Cho-containing metabolites at the end of the pulse period were quite similar for all of the strains, it seems that Sec14p can override its two homologs regarding their potential direct effects on Cho uptake and PC synthesis through the CDP-Chol pathway. To test this hypothesis the activity of the Cho transporter was determined in SEC14, SFH2, and SFH4 strains in a sec14Δ background under two different growth conditions: at 25 °C (Sec14p-proficient) or after 3 h at 37 °C (Sec14p-deficient) (Table IV). These three strains took up Cho at comparable rates when grown at 25 °C. However, after 3 h at the restrictive temperature for the sec14Δ allele, the activity of the Cho transporter measured in SFH2 and SFH4 cells was reduced to 75 and 60% that of cells containing SEC14, respectively.

Previous work illustrated that an extracellular Cho concentration above 100 μM was associated with a switch from Pct1p as the rate-limiting step to choline kinase being the major regulator for PC synthesis (9). Because cellular Cho content was clearly altered in yeast strains expressing SFH2 and SFH4

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**Table III**

| Relevant genotype | Growth rate<sup>a</sup> | Choline uptake<sup>b</sup> | Choline content<sup>c</sup> |
|------------------|------------------------|-----------------------------|-----------------------------|
|                  | min<sup>-1</sup>       | nmol Cho min<sup>-1</sup> mg protein<sup>-1</sup> | pmol Cho OD<sub>540</sub><sup>-1</sup> |
| SEC14            | 3.7 ± 0.6<sup>b</sup> | 1.18 ± 0.02                  | 123 ± 82                   |
| SEC14K66,239A    | 3.6 ± 0.9<sup>b</sup> | 1.32 ± 0.03                  | ND<sup>d</sup>             |
| SFH2             | 4.0 ± 0.8<sup>b</sup> | 0.87 ± 0.04                  | 876 ± 118                  |
| SFH4             | 4.5 ± 0.2<sup>b</sup> | 0.31 ± 0.02                  | 817 ± 263                  |

<sup>a</sup> Growth rate was calculated for logarithmically growing cells in synthetic minimal glucose medium at 25 °C.

<sup>b</sup> Cho uptake measurement was performed in logarithmically growing cells suspended in minimal glucose medium containing 10 μM (10,000 dpm/nmol) [14C]Cho. At 2 and 4 min uptake was stopped, and the cells were harvested immediately by filtration on glass microfiber filters. Cells were washed thoroughly under vacuum, and the associated label was determined by liquid scintillation counting. Data represent the mean ± S.E. of two independent determinations performed in triplicate.

<sup>c</sup> Intracellular Cho content was determined in yeast cells growing at 25 °C to mid log phase in minimal supplemented glucose medium. After lipid extraction the aqueous partitioning metabolites were recovered and filtered through a Dowex AG 1-X8 column. The amount of Cho contained in the flow-through was estimated enzymatically. Data represent the mean ± S.E. of four independent determinations.

<sup>d</sup> Not determined.
versus SEC14 (Table III), we analyzed the P-Cho:Cho ratios calculated from the data presented in Fig. 3 to assess the regulatory roles of Pct1p versus choline kinase in these cells. The P-Cho:Cho ratios for SFH2- and SFH4-containing cells at every time point during the chase at 25 and 37 °C differed markedly from those calculated for the SEC14 and SEC14K66,239A strains (23.7 ± 2.3, 24.6 ± 3.5, 10.2 ± 1.5, and 9.9 ± 1.7 nCi/10^7 cells for pRS413-SEC14, YCp-HIS3-SEC14K66,239A, YEp-URA3-SFH2, and YEp-URA3-SFH4 sec14 strains, respectively). Data are expressed as a percentage of total label recovered in each fraction at each time point after the shift to unlabeled medium. This experiment was repeated three times with qualitatively similar results.

**TABLE IV**

Choline uptake in sec14" background

| Relevant genotype | Choline uptake  | Choline uptake  |
|-------------------|----------------|----------------|
|                   | 25 °C          | 37 °C          |
| Choline uptake    | n mol Cho min⁻¹ | n mol Cho min⁻¹ |
| mg protein        |                |                |
| SEC14             | 1.52 ± 0.03    | 1.34 ± 0.11    |
| SFH2              | 1.38 ± 0.05    | 0.97 ± 0.06    |
| SFH4              | 1.32 ± 0.02    | 0.82 ± 0.02    |
| Empty vector      | 1.43 ± 0.06    | 0.72 ± 0.04    |

Cells growing at 25 or 37 °C in minimal glucose medium were pelleted, washed twice in fresh medium, and resuspended in fresh medium containing 10 μM [14C]choline. At 2 and 4 min uptake was stopped, and choline uptake was determined as described under "Experimental Procedures."

**FIG. 3.** Decreased GPCho accumulation in Sec14p-deficient strains at 25 and 37 °C. The sec14Δ strain containing the following plasmids: pRS413-SEC14, YCp-HIS3-SEC14K66,239A, YEp-URA3-SFH2, and YEp-URA3-SFH4, was cultured at 25 °C to mid log phase in minimal glucose medium containing the required supplements for cell growth. Cells were harvested, washed, and resuspended in the same medium containing radiolabeled Cho for 30 min at 25 °C. At time zero, cells were harvested, washed, and resuspended in identically supplemented medium containing 10 mM nonradioactive Cho warmed at 25 °C (A) or at 37 °C (B). At the indicated times, aliquots of the culture were withdrawn and analyzed for radioactivity distributed among the indicated Cho-containing metabolites. Total radioactivity taken up by SFH2 and SFH4 strains was about one-third relative to the label incorporated in SEC14 and SEC14K66,239A strains (23.7 ± 2.3, 24.6 ± 3.5, 10.2 ± 1.5, and 9.9 ± 1.7 nCi/10^7 cells for pRS413-SEC14, YCp-HIS3-SEC14K66,239A, YEp-URA3-SFH2, and YEp-URA3-SFH4 sec14 strains, respectively). Data are expressed as a percentage of total label recovered in each fraction at each time point after the shift to unlabeled medium. This experiment was repeated three times with qualitatively similar results.
cells are likely incapable of regulating PC synthesis at this step. At longer chase times for SFH2- and SFH4-expressing cells the P-Cho:Cho ratio was ~2, but these quotients were not the trivial result of two decreasing values as the chase progressed. Instead, at 30, 60, and 90 min the label associated with Cho and P-Cho exhibited low but significant values above background. The Cho concentration of the chase medium (10 mM) precluded the possibility that the label associated with intracellular Cho at any chase time point could have been derived from labeled Cho produced by Spo14p and simultaneously excreted from the cells, mixed with the unlabeled Cho of the chase medium, and taken up again. These data tempt us to speculate that steady specific activity for both Cho and P-Cho observed during the chase period at 25 and 37 °C for SFH2 and SFH4 cells was the result of the intracellular release of Cho generated through a high turnover of labeled PC mediated by Spo14p together with reduced uptake of unlabeled Cho from the chase medium (see Table III). To analyze further the hypothesis that Sfh2p- and Sfh4p-overexpressing cells exhibit decreased Pct1p regulation of PC synthesis we performed straight radiolabeled choline pulse experiments for up to 90 min. Consistent with the pulse-chase experiments, in sec14Δ cells we observed that the P-Cho:Cho ratio equilibrated at ~8 after 30 min in SEC14- and SEC14K66,239A-containing cells, whereas in Sfh2p- and Sfh4p-overexpressing cells the ratio remained ~2 for the entire pulse period (data not shown).

We had observed that SFH2- and SFH4-containing cells excreted labeled Cho against 10 mM Cho-containing medium. To determine whether the excretion of Cho correlated with an altered intracellular Cho concentration we measured Cho mass in SEC14, SFH2, and SFH4 cells in a sec14Δ background. Sfh2p- and Sfh4p-overexpressing cells contained about 2.5-fold the amount of Cho contained in Sec14p-proficient cells (Table III).

**Fig. 4.** Altered P-Cho:Cho ratio in SFH2- and SFH4-overexpressing cells. The P-Cho:Cho ratio during the chase period at 25 °C (A) or at 37 °C (B) was calculated for each time point from the experimental data shown in Fig. 3.

**DISCUSSION**

We present evidence that Nte1p, an endoplasmic reticulum integral membrane phospholipase that exhibits a restricted specificity for PC derived from the CDP-Cho pathway, functionally interacts with Sec14p. When Pct1p, upon Sec14p inactivation, is released from its control the flux through the CDP-Cho pathway increases depending on the availability of Cho and...
diacylglycerol (7, 20–22, 31, 32). It might be expected that a local augmented PC content would induce Nte1p promoting an increased rate of PC degradation. This is not the case because the biochemical data clearly indicate that the accumulation of intracellular GPCho, the product of Nte1p activity, takes place when Sec14p is functional. Nte1p and Pet1p are targets of Sec14p and Sec14p^{K66,239A}, an engineered isofrom devoid of in vitro PI transfer but still competent for PC transfer activity (33). Sec14p bound to PC, among other tasks, promotes vesicular transport controlling the amount of PC derived from the CDP-Cho pathway present at certain critical subcellular locations. Sec14p exerts this function by regulating the rate of PC biosynthesis through the CDP-Cho pathway (7) and increasing its degradation by Nte1p. Consistent with this role we observed that overexpression of Nte1p enabled growth of sec14 cells at the restrictive temperature of 35 °C. The PC turnover experiments and Cho uptake measurements performed in sec14 background provide evidence that Sec14p exerts a central role controlling PC metabolism, which is not affected by the overexpression of Shh2p or Shh4p nor restored by them when Sec14p is destabilized. At 25 °C total label uptake measurements during the pulse period of the pulse-chase experiment, as well as Cho transport activities, indicate that Cho import from the medium is not affected by the overexpression of Shh2p and Shh4p in Sec14p-proficient cells. The turnover profile at 37 °C of Shh2p-overexpressing cells was almost identical to the sec14 strain. SFH4 cells exhibited a similar profile (low intracellular GPCho, high extracellular Cho) with the excretion of Cho more pronounced, probably related to the Spo14p dependence of the mechanism mediated by Shh4p to complement the absence of Sec14p. At 37 °C SFH2 and SFH4 are viable, whereas sec14 is not, indicating that overexpression of Shh2p and Shh4p rescues the cells from the absence of Sec14p through a mechanism that does not restore the profile of PC turnover corresponding to the Sec14p-proficient cell. The analyses performed in SFH2- and SFH4-overexpressing cells in a sec14Δ background revealed a profound alteration of PC metabolism triggered by the absence of Sec14p: Nte1p unresponsiveness, Spo14p activation, and deregulation of Pet1p activity. In Sec14p-deficient cells rescued by the overexpression of Shh2p and Shh4p new equilibriums involving PC metabolism are reached to allow cell viability. Sec14p thus modulates the PC derived from the CDP-Cho pathway by simultaneous inhibition of the flux at the Pet1p-catalyzed step as well as increased PC deacylation by Nte1p. On the other hand, SFH2 and SFH4 cells devoid of Sec14p do not regulate the flux through this pathway at the condensation step catalyzed by Pet1p as is observed in Sec14p-proficient cells. In addition, CDP-Cho pathway-derived PC is only marginally degraded by Nte1p when Sec14p is absent; instead, a high turnover of PC mediated by Spo14p is evoked as in these cells the intracellular Cho content is higher than in Sec14p-proficient cells. The metabolic data indicate that the massive PC turnover mediated by Spo14p contributes to the observed increase in intracellular Cho content. Total choline uptake measurements performed in SFH2 and SFH4 cells for the pulse-chase experiments in SEC14-deficient cells revealed that choline uptake was approximately one-third that of SEC14 and SEC14^{K66,239A} cells. Consist with this observation were measurements of the activity of the Cho transporter, which was dramatically reduced in SFH4 cells but not in SFH2 cells. Finally, Shh2p- and Shh4p-overexpressing cells excreted Cho into medium containing 10 mM Cho. The total data suggest that SFH2 and SFH4 cells regulate the Cho excretion, but the mechanism underlying this excretion process is unknown (20). One possibility is that Spo14p by itself or associated with another unknown partner at the plasma membrane actively pumps Cho molecules out of the cell as they are produced; however, the combined metabolic and choline mass data presented in this study favor an explanation whereby Cho molecules formed by Spo14p activity are released into the cytoplasm where they mix with the intracellular pool. Cho released by turnover of PC by Spo14p can be used for PC synthesis by the CDP-Cho pathway. However, extensive PC turnover by Spo14p results in active Cho excretion from the cell as a mechanism to regulate substrate supply for the CDP-Cho pathway.
Golgi-derived vesicular transport processes with Spo14-mediated PC turnover acting as a positive regulator of Sec14p-mediated vesicular transport (21, 22) and PC synthesis through the CDP-Cho pathway negatively regulating of this same vesicular transport pathway (4, 31). Sec14p was known to regulate PC homeostasis actively by inhibiting the rate-limiting Pct1p-catalyzed step for PC synthesis (7), and beyond the same vesicular transport pathway (4, 31). Sec14p was known to regulate PC homeostasis negatively regulating of this mediated vesicular transport (21, 22) and PC synthesis (26, 27, 28, 29). We also describe a second role for Sec14p in mediating new PC metabolic control features we ascribe to Sfh2p and Shf4p, we also describe a second role for Sec14p in mediating PC homeostasis with Sec14p acting as a positive regulator of Nte1p-mediated PC deacylation.

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