A Role for Phospholipase D (Pld1p) in Growth, Secretion, and Regulation of Membrane Lipid Synthesis in Yeast*

(Received for publication, March 6, 1998, and in revised form, May 1, 1998)

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The SEC14 gene encodes a phosphatidylinositol/phosphatidylcholine transfer protein essential for secretion and growth in yeast (1). Mutations (cki1, cct1, and cpt1) in the CDP-choline pathway for phosphatidylcholine synthesis suppress the sec14 growth defect (2), permitting sec14ts cki1, sec14ts cct1, and sec14ts cpt1 strains to grow at the sec14ts restrictive temperature. Previously, we reported that these double mutant strains also excrète the phospholipid metabolites, choline and inositol (3). We now report that these choline and inositol excrètion phenotypes are eliminated when the spo14 (PLD1) gene encoding phospholipase D1 is deleted. In contrast to sec14ts cki1 strains, sec14ts cki1 pld1 strains are not viable at the sec14ts restrictive temperature and exhibit a pattern of invertase secretion comparable with sec14ts strains. Thus, the PLD1 gene product appears to play an essential role in the suppression of the sec14ts defect by CDP-choline pathway mutations, indicating a role for phospholipase D1 in growth and secretion. Furthermore, sec14ts strains exhibit elevated Ca2+-independent, phosphatidylinositol 4,5-bisphosphate-stimulated phospholipase D activity. We also propose that phospholipase D1-mediated phosphatidylcholine turnover generates a signal that activates transcription of INO1, the structural gene for inositol 1-phosphate synthase.

In mammalian cells, phospholipase D (PLD) and/or its primary lipid product, phosphatidic acid (PA), are involved in regulating a number of vital cellular functions, including cytoskeleton formation, membrane trafficking, and the secretory process (4–9). Surprisingly, given the important roles ascribed to PLD in mammalian cells, mutants carrying a deletion of the gene encoding all of the measurable Ca2+-independent, PIP2-dependent phospholipase D activity in yeast are viable (10–12). However, pld1 mutants proved to be allelic to spo14 mutants, originally identified on the basis of their inability to undergo meiosis and sporulation (13, 14).

Whereas PA has been postulated to play a role in membrane trafficking and secretion in mammalian cells (15), Kearns et al. (16) proposed that diacylglycerol (DAG) plays a central role in the secretory process in yeast. In support of this hypothesis, Kearns et al. (16) reported that the sec1-29ts mutation leads to elevated DAG levels, thereby suppressing the growth and secretion phenotypes of mutants lacking functional Sec14p, the phosphatidylinositol (PI) phosphatidylcholine (PC) transfer protein that is required for growth and secretion in yeast (1). The sec14 growth phenotype is also suppressed by mutations in the CDP-choline pathway for PC biosynthesis (2, 17).

We recently discovered that sec14ts strains carrying CDP-choline pathway mutations (i.e. sec14ts cki1, sec14ts cct1, and sec14ts cpt1) excrete both inositol and choline when elevated to the sec14ts restrictive temperature (37 °C). The excretion of inositol is caused by derepression of INO1, structural gene for inositol 1-phosphate synthase. The excretion of choline results from accelerated turnover of PC under circumstances where choline reutilization is blocked (i.e. in the presence of a CDP-choline pathway mutation) (3). Free choline and PA are produced when PC is hydrolyzed by the action of PLD (18). In the sec14ts cki1 strain elevated to the sec14ts restrictive temperature, 50% of the total cellular PC is turned over per generation via a PLD-mediated route (3). In the present study, we asked whether Pld1p was responsible for this elevated PC turnover and whether PLD1-mediated PC turnover is necessary for the suppression by cki1 and other CDP-choline pathway mutations of the sec14ts growth and secretion defects.

EXPERIMENTAL PROCEDURES

Yeast Strains—A disruption of the PLD1 (SPO14) gene (pld1::URA3), as described by Waksman et al. (19) was made in a sec14ts cki1 strain (genotype Mata ura3 his3 lys2 sec14-3°C cki1::HIS3) (from V. Bankaitis), using plasmid pPLD::URA3 (from M. Lisecvitch). The triple mutant (Mata ura3 his3 lys2 sec14-3°C cki1::HIS3 pld1::URA3) was crossed to wild type strain JAGWT (Mata SEC14 CKI1 PLD1 ura3 his3 trp1 leu2) (20), and tetrad analysis was performed.

Assay for Opc—An Opc assay—Strains were grown on plates containing complete synthetic medium lacking inositol and choline (1°C medium) at 30 °C. Tester strains auxotrophic for choline or inositol, respectively, were sprayed onto the plates that were then incubated for 2 additional days at 30°C (21). A halo of growth around the strains indicates choline excrètion (Opc−) or inositol excrètion (Opc+), respectively (see Fig. 1).

Phospholipase D Assay—Yeast strains were grown to mid-logarithmic phase in I°C medium at 30°C. Extracts were prepared, and phospholipase D1 activity was measured using the method of Waksman et al. (19) at 30°C for 60 min using C6-NBD-PC (Avanti Polar lipids) as substrate in the presence of 50 mm HEPES, pH 7.0, Pi, EDTA, and EGTA.

Invertase Assay—Strains were grown in 1% yeast extract, 2% Bactopectone, 3% glucose (YPED) medium at 25°C until mid-logarithmic phase of growth. At this time, they were shifted to low glucose YEP medium for 1 h at 25 or 37°C. The secretion index, defined as the percentage of total cellular invertase activity that is secreted (i.e. external to the cell) was determined (16).

Lipid Analysis—Strains were grown in the presence of H23PO4 (10 μC/cm) to uniformly labeling at 30°C in 1°C medium and were then
chased in unlabeled medium for 4 h at 37 °C. Phospholipids were extracted and separated as described previously (3). To measure total lipids, yeast strains were grown to mid-logarithmic phase (A660 = 0.6–0.8) in synthetic medium containing 2 μCi/ml [125I]-labeled acetate. Lipids were extracted, separated by TLC, and detected by fluorography, and the bands were scraped and counted (22).

RESULTS

A SPO14 (PLD1) deletion (pld1:ura3) was created in the sec14<sup>ts</sup> cki1 strain, producing the triple mutant, sec14<sup>ts</sup> cki1 pld1. The triple mutant, unlike its sec14<sup>ts</sup> cki1 parent but similar to sec14<sup>ts</sup> strains, failed to grow at 37 °C. At 30 °C, the semi-permissive temperature, the triple mutant also lacked the inositol excretion (Opi<sup>−</sup>) and choline excretion (Opc<sup>−</sup>) phenotypes that are observed in the sec14<sup>ts</sup> cki1 strain (Fig. 1). We crossed the triple mutant to a wild type strain and 21 complete tetrads from the cross were evaluated. The Opi<sup>−</sup> and Opc<sup>−</sup> phenotypes of all eight possible genotypes involving the three allelic pairs in eight spores of two tetrads from this cross are depicted in Fig. 1. All sec14<sup>ts</sup> cki1 strains (10 independent segregants) were able to grow at 37 °C and had the Opi<sup>−</sup> and Opc<sup>−</sup> phenotypes at 30 °C as reported previously for strains of the sec14<sup>ts</sup> cki1 genotype (3). In contrast, all sec14<sup>ts</sup> cki1 pld1 strains (11 independent segregants) were unable to grow at 37 °C and furthermore lacked the Opi<sup>−</sup> and Opc<sup>−</sup> phenotypes at 30 °C (Fig. 1). In 35 tetrads analyzed from a cross of a sec14<sup>ts</sup> pld1 to a sec14<sup>ts</sup> cct1 strain, all sec14<sup>ts</sup> cct1 strains tested exhibited Opi<sup>−</sup> and Opc<sup>−</sup> phenotypes at 30 °C, whereas all sec14<sup>ts</sup> cct1 pld1 strains tested were unable to grow at 37 °C and lacked Opi<sup>−</sup> and Opc<sup>−</sup> phenotypes at 30 °C (data not shown).

The Opi<sup>−</sup> phenotype exhibited by sec14<sup>ts</sup> cki1 strains at both 30 °C (see Fig. 4) and 37 °C (3) is associated with misregulation of inositol-1-phosphate synthase (21), product of the INO1 gene.

Regulation of the INO1 gene was analyzed in all of the strains shown in Fig. 1 by measuring expression of β-galactosidase from an INO1 lacZ promoter gene fusion as described previously (3). Except for sec14<sup>ts</sup> cki1, all of the strains shown in Fig. 1 exhibited patterns of INO1 expression at 30 °C similar to that of the wild type strain. In the sec14<sup>ts</sup> cki1 strain grown at 30 °C, INO1 lacZ expression was not fully repressed in the absence of inositol (58 ± 12 units of β-galactosidase in sec14<sup>ts</sup> cki1 versus 15 ± 6 units in wild type) and was greatly overexpressed in the absence of inositol (407 ± 34 units in sec14<sup>ts</sup> cki1 versus 148 ± 7 units in wild type). Deletion of the PLD1 gene in the sec14<sup>ts</sup> cki1 genetic background (i.e. to produce the sec14<sup>ts</sup> cki1 pld1 strain) simultaneously eliminated the Opi<sup>−</sup> phenotype at 30 °C (Fig. 1) and restored INO1 expression to a pattern similar to wild type (183 ± 12 units of β-galactosidase in the absence of inositol; 13 ± 1 units in the presence of inositol).

Pld1p Is Required for Growth and Viability in the sec14<sup>ts</sup> cki1 Genetic Background—The sec14<sup>ts</sup> cki1 pld1 strain stopped growing within one doubling time upon elevation to the restrictive temperature of 37 °C in a fashion identical to the sec14<sup>ts</sup> strain (Fig. 2). The sec14<sup>ts</sup> cki1 pld1 and sec14<sup>ts</sup> strains both lost viability at 37 °C (data not shown). In contrast, the sec14<sup>ts</sup> cki1 strain continued to grow when shifted to 37 °C. Strains carrying the pld1 or cki1 mutations, singly or in combination (i.e. cki1 pld1) without the sec14<sup>ts</sup> mutation, continued to grow when shifted to 37 °C. The sec14<sup>ts</sup> pld1 strain also stopped growing and lost viability at 37 °C, similar to strains of the sec14<sup>ts</sup> and sec14<sup>ts</sup> cki1 pld1 genotype (data not shown).

The PLD1 Deletion Affects Secretory Function in the sec14<sup>ts</sup> cki1 Genetic Background—Indicative of a defect in secretion, as previously reported (23), the sec14<sup>ts</sup> strain exhibited a very low ratio of external versus total invertase when shifted to low glucose medium at 37 °C (Fig. 3A). The sec14<sup>ts</sup> cki1 strain that grows quite well when shifted to 37 °C (Fig. 2, A and B) exhibited only partial recovery of the secretory function in 1 h after the shift to 37 °C low glucose medium (Fig. 3A). The sec14<sup>ts</sup> cki1 strain also showed altered kinetics of invertase derepression (data not shown), including overexpression relative to wild type and retention of a large internal pool of invertase (Fig. 3B). However, the sec14<sup>ts</sup> cki1 pld1 strain resembled the sec14<sup>ts</sup> rather than the sec14<sup>ts</sup> cki1 strain in its profile of invertase expression and retention in the cellular pool (Fig. 3, A and B).

Phospholipase D Activity Is Elevated in sec14<sup>ts</sup> Mutants—The cki1 and wild type strains grown at 30 °C had similar levels of PLD activity, whereas the strains carrying the sec14<sup>ts</sup> mutation (pld1), which is a constitutive mutant for PLD, exhibited 20- to 30-fold higher phospholipase D activity than wild type and cki1 strains (Fig. 2, C and D). The pkc1 mutant, which has elevated PLD activity, exhibited 5- to 6-fold higher phospholipase D activity than wild type cells (Fig. 2, C and D). The sec14<sup>ts</sup> cki1 pld1 strain resembled the sec14<sup>ts</sup> rather than the sec14<sup>ts</sup> cki1 strain in its profile of invertase expression and retention in the cellular pool (Fig. 3, A and B).
allele (sec14ts and sec14ts chi1) both exhibited an increase of about 50% in Ca2+-independent, PIP2-stimulated PLD activity compared with wild type (Fig. 4A). This result is consistent with the appearance of an enlarged choline excretion ring in the sec14ts chi1 strain, as compared with the chi1 strain, as well as the small choline excretion ring in sec14ts versus no ring at all in wild type (Fig. 1). Sec14p has already been reported to have a negative regulatory effect upon the activity of the CCT1 gene product (24). Our results suggest that Sec14p also affects Pld1p regulation.

Unlike the sec14ts chi1 pld1 strain, the chi1 pld1 double mutant exhibited a residual Opc phenotype (Fig. 1). This result suggests that at least some of the turnover of PC that is responsible for the Opc phenotype in the chi1 genetic background is not mediated by Pld1p. Pld1p-independent activity that does not require PIP2 is Ca2+-dependent, has no detectable transphosphatidylation activity, and utilizes both phosphatidylserine and phosphatidylethanolamine in preference to PC has been reported (19, 25). Thus, the residual choline excretion seen in chi1 strains in which the PLD1 gene has been deleted (i.e. chi1 pld1 genotype; Fig. 1) could be the result of an as yet unidentified PLD activity. The existence of Pld1p-independent activity may explain how yeast cells can survive the deletion of the PLD1 gene. We observed that the sec14ts chi1 pld1 strain lacked not only the enhancement of the Opc phenotype, seen in the sec14ts chi1 strain as compared with the chi1 strain, but lacked the Opc phenotype entirely (Fig. 1). The enzyme activity responsible for the residual Opc phenotype observed in the chi1 pld1 strain might, therefore, also be under some form of control by Sec14p.

Pld1p Affects Phospholipid Metabolism in Vivo—To study phospholipid turnover in vivo, we labeled the lipids to steady-state with H3-32PO4 at 30 °C and then shifted the cells to unlabeled medium at the restrictive temperature of 37 °C for 4 h to chase the radioactive phosphate (Fig. 4B). In wild type cells, the label associated with PA decreased as a proportion of total lipid associated 32P during the chase following the temperature shift, and PC continued to acquire label throughout the chase (Fig. 4B). In sec14ts chi1 cells, however, the proportion of 32P label associated with PA at steady-state started slightly higher than in wild type cells and actually increased during the chase at 37 °C. Furthermore, unlike the pattern observed in wild type, the proportion of label associated with PC declined slightly in the sec14ts chi1 strain, indicating that PC was being broken down at a rate that exceeded its accumulation of label from previously labeled precursors, such as PE. The increase in the proportion of label in PA at 37 °C (S) and were then chased with unlabeled medium for 4 h at 37 °C (C). Phospholipids were extracted and separated as described previously (3). Data represent the averages of three experiments ± S.D. White bars, wild type; hatched bars, sec14ts chi1; black bars, sec14ts chi1 pld1.

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The enzyme activity responsible for the residual Opc phenotype observed in the chi1 pld1 strain might, therefore, also be under some form of control by Sec14p.
difference among any of the strains examined at 30 or 37 °C in the proportion of DAG (data not shown).

DISCUSSION

We have demonstrated several functional consequences of PLD1 deletion in mitotically dividing yeast cells. These findings have important implications for the use of Saccharomyces cerevisiae as a model relevant to the study of the cellular roles of the various forms of PLD, and of lipid turnover in general, in the secretory pathway and in mitotic cell division in higher eukaryotes. Specifically, we have demonstrated that the high level of PC turnover, producing the choline excretion phenotype previously documented in sec14A cki1 cells growing at 37 °C (3), requires functional Pld1p (Fig. 1). Our results also suggest that Pld1p is activated upon inactivation of Sec14p, the yeast PI/PC transporter (Fig. 4A). Moreover, the residual Pld1p-independent PLD activity, responsible for the choline excretion observed in cells of the cki1 pld1 genotype (Fig. 1), appears to be inactivated in sec14A cki1 pld1 strains. This regulatory interplay between Sec14p and PLD has consequences for mitotic growth and secretion (Figs. 2 and 3). sec14A mutants shifted to the restrictive temperature arrest in the secretory process at the late Golgi stage (23). The SEC14 gene product is a lipid transfer protein capable of binding both PI and PC (1). The precise role of Sec14p in the secretory process has remained elusive as has the mechanism by which CDP-choline pathway mutations suppress the SEC14 growth defect. Skinner et al. (24) demonstrated that Sec14p regulates the CCT1 gene product, which catalyzes the rate-limiting step of the CDP-choline pathway. They proposed that this regulation plays a crucial role in maintaining cellular DAG levels, a conclusion supported by recent results of Kearns et al. (16). In the present study, we observed that sec14A cki1 cells shifted to 37 °C exhibit an abnormal pattern of secretion involving high internal and external invertase expression (Fig. 3), suggesting that the mechanism of suppression of the sec14 defect by the cki1 mutation does not fully restore a wild type pattern of secretion. The abnormal pattern of invertase expression and secretion observed in the sec14A cki1 strain is eliminated by the pld1 deletion mutation (Fig. 3) and, therefore, must be dependent on Pld1p. Moreover, the abnormal pattern of secretory activity supported by elevated PLD1 activity appears to be a necessary component of the mechanism of suppression of sec14A by CDP-choline mutations because sec14A cki1 pld1 cells cannot grow at 37 °C. Consistent with our observations, there are many reports in mammalian cells of a link between PLD activity and secretory function (4), 9, including a recent demonstration that purified human PLD1 can stimulate release of nascent secretory vesicles from the trans-Golgi network in permeabilized cells (7).

Deletion of the PLD1 gene in the sec14A cki1 genetic background also eliminates the Opi- phenotype (Fig. 1) and restores INO1 regulation to the wild type pattern. INO1 is the most highly regulated of the group of coregulated genes of phospholipid biosynthesis, which contain a repeated element, UASINO, in their promoters (26). We propose that the misregulation of the INO1 gene in sec14A cki1 cells shifted to 37 °C (3) is the result of elevated Pld1p driven PC turnover. Because we have previously demonstrated that free choline is not responsible for the signal controlling INO1 repression/derepression (27), we suspect that PA production is directly or indirectly responsible. Notably, PA has been implicated in both signal transduction and membrane trafficking in mammalian cells (4). We speculate that the signal, activating INO1 transcription upon shift of the sec14A cki1 strain to 37 °C, most likely originates in the Golgi where Sec14p localizes (2). The INO1 gene has recently been shown to be regulated in response to the accumulation of unfolded proteins in the endoplasmic reticulum (ER) by increasing ER localized chaperones (29). Thus, INO1 appears to be regulated in response to signals involving membrane biogenesis in both the ER and Golgi.

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