Effect of Growth Phase on Phospholipid Biosynthesis in *Saccharomyces cerevisiae*[†]

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The effect of growth phase on the membrane-associated phospholipid biosynthetic enzymes CDP-diacylglycerol synthase, phosphatidylserine synthase, phosphatidylinositol synthase, and the phospholipid *N*-methyltransferases in wild-type *Saccharomyces cerevisiae* was examined. Maximum activities were found in the exponential phase of cells grown in complete synthetic medium. As cells entered the stationary phase of growth, the activities of the CDP-diacylglycerol synthase, phosphatidylserine synthase, and the phospholipid *N*-methyltransferases decreased 2.5- to 5-fold. The subunit levels of phosphatidylserine synthase and the cytoplasmic-associated enzyme inositol-1-phosphate synthase were not significantly affected by the growth phase. When grown in medium supplemented with inositol-choline, cells in the exponential phase of growth had reduced CDP-diacylglycerol synthase, phosphatidylserine synthase, and phospholipid *N*-methyltransferase activities, with repressed subunit levels of phosphatidylserine synthase and inositol-1-phosphate synthase compared with cells grown without inositol-choline. Enzyme activity levels remained reduced in the stationary phase of growth of cells supplemented with inositol-choline. The phosphatidylserine synthase and inositol-1-phosphate synthase subunit levels, however, were derepressed. Phosphatidylinositol synthase (activity and subunit) was not affected by growth in medium supplemented with or without inositol-choline or the growth phase of the culture. The phospholipid composition of cells in the exponential and stationary phase of growth was also examined. The phosphatidylinositol to phosphatidylserine ratio doubled in stationary-phase cells. The phosphatidylcholine to phosphatidylethanolamine ratio was not significantly affected by the growth phase of cells.

A major component of biological membranes is the phospholipids. The synthesis of phospholipids and their assembly into cellular membranes are major activities that cells engage in throughout their existence (29). The generally accepted pathway for the synthesis of phospholipids in *Saccharomyces cerevisiae* is shown in Fig. 1. Phospholipid biosynthesis in *S. cerevisiae* (18) is carried out by enzymes common to higher eucaryotic organisms (14). One exception is that in *S. cerevisiae*, phosphatidylserine (PS) is synthesized from CDP-diacylglycerol (CDP-DG) and serine (2), whereas in mammals PS is synthesized via an exchange reaction between phosphatidylethanolamine (PE) and serine (25). The phospholipid biosynthetic enzymes in *S. cerevisiae* are associated with the mitochondrial and microsomal fractions of the cell (27).

The biosynthesis of phosphatidylcholine (PC) by the reaction sequence phosphatidic acid (PA) → CDP-DG → PS → PE → PC is coordinately regulated to the synthesis of phosphatidylinositol (PI) through the control of inositol synthesis (20). The enzyme activities leading to the formation of PC, namely CDP-DG synthase (21), PS synthase (10, 26), PS decarboxylase (11), and the phospholipid *N*-methyltransferases (PNMTs) (35, 36) are reduced in exponentially growing wild-type cells when the medium is supplemented with inositol-choline. CDP-DG synthase (21) and PS synthase (30) activities are also reduced when wild-type cells are grown in medium containing inositol-ethanolamine.

In the absence of inositol, choline or ethanolamine has no effect on CDP-DG synthase (21), PS synthase (30), or PNMT (26, 36) activities. The enzymes of the auxiliary CDP-choline-dependent pathway for the synthesis of PC are constitutive (22). Unlike the enzymes involved in the primary route of PC synthesis, PI synthase is not regulated in response to water-soluble phospholipid precursors (15, 26). The cytoplasmic-associated enzyme inositol-1-phosphate synthase (which is responsible for the synthesis of inositol), however, is repressed in cells grown in the presence of inositol (12, 13) and inositol-choline (26).

Results of previous studies (23, 33) have shown that total phospholipid synthesis in *S. cerevisiae* decreases as cells enter the stationary phase of growth, whereas the synthesis of total triacylglycerols increases. The increase in triacylglycerol synthesis in the stationary phase of growth is believed to be a result of the induction of PA phosphatase activity (23). PA is converted to CDP-DG for phospholipid synthesis or converted to DG for triacylglycerol synthesis (Fig. 1). The effect of the growth phase on the regulation of the phospholipid biosynthetic enzymes is not known and is the subject of this report. We analyzed the levels of activity and enzyme subunits of several key phospholipid biosynthetic enzymes during the growth of *S. cerevisiae*.

MATERIALS AND METHODS

Materials. CTP, L-serine, choline, *myo*-inositol, S-adenosyl-L-methionine, phospholipids, Ponceau S solution, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, Mo.). [5-³H]CTP, L-[3-³H]serine, S-adenosyl-L-[methyl-³H]methionine, and ³²P_i were obtained

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FIG. 2. Phospholipid biosynthetic enzyme activities during growth. *S. cerevisiae ade5* was grown in complete synthetic medium in the absence (A) and presence (C) of inositol-choline. Cells were harvested at the indicated time intervals, and cell extracts were prepared as described in the text. (A and C) Cell number (●) was determined by counting under a microscope on a hemacytometer. (B and D) Activities (in units per milligram) of CDP-DG synthase (■); PS synthase (○); PNMTs (□); and PI synthase (●) were calculated from triplicate determinations.

Electrophoresis and immunoblotting. Cell extracts were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate with 10% polyacrylamide slab gels (28). Proteins were transferred electrophoretically to unmodified nitrocellulose papers (pore size, 0.2 μ m), as described by Burnette (6) with the modifications described by Haid and Suissa (17). The transfer buffer contained 25 mM Tris (pH 8.3), 192 mM glycine, 20% methanol, and 0.02% sodium dodecyl sulfate. The transfer was performed for 1 h at 100 V with a Hoefer TE 52 Transphor unit. Protein transfer was verified by staining the nitrocellulose papers with a 0.2% Ponceau S solution in 3% trichloroacetic acid–3% sulfosalicylic acid. The dye was completely removed from the proteins by incubating the papers for 60 min in TBS (20 mM Tris hydrochloride [pH 7.5] and 500 mM NaCl) containing 3% nonfat dry milk (24). The nitrocellulose papers were incubated overnight at room temperature in a 1:50 dilution of PS synthase antiserum or PI synthase antiserum or in a 1:5,000 dilution of inositol-1-phosphate synthase antiserum in TBS, using 1% nonfat dry milk as a blocking agent (24). The nitrocellulose papers were washed with TBS containing 0.05% Tween 20 followed by washing with TBS. The papers were incubated at room temperature for 1 h in a 1:3,000 dilution of goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase in TBS containing 1% nonfat dry milk. Papers were washed with TBS followed by a 30-min incubation with a freshly prepared solution of 0.05% 4-chloro-1-naphthol–0.015% hydrogen peroxide in TBS for color development (34). The immunoblot

signals were optimized by analyzing a number of antigen and antibody concentrations and were in the linear range of detectability.

Phospholipid composition analysis. The phospholipid composition of cells was determined as described previously (1), with some modifications. Cultures of 5 ml were grown in the indicated medium in the presence of 50 μ Ci of 32 P_i. Cells were harvested by centrifugation at exponential and stationary phases of growth, washed with distilled water, and suspended in 3 ml of chloroform-methanol-water (1:2:0.8; vol/vol/vol). The cell suspension was mixed with glass beads (ca. 0.05 g) and vortexed intermittently for 1 h at 23°C. Phospholipids were extracted from the broken cell suspension by the method described by Bligh and Dyer (4). The chloroform phase was dried in vacuo, and the residue was dissolved in a minimum volume of chloroform-methanol (1:1). A portion of the extract was counted by liquid scintillation to determine the total lipid extractable counts. The remaining lipids were separated by two-dimensional paper chromatography with EDTA-treated SG81 paper (Whatman, Inc., Clifton, N.J.) as described by Steiner and Lester (32). Dimension 1 was chloroform-methanol–30% ammonium hydroxide–water (66:27:3:0.8) and dimension 2 was chloroform-methanol-glacial acetic acid–water (32:4:5:1). The positions of the labeled phospholipids on the chromatograms were determined by autoradiography. The identity of each phospholipid was determined by comparing its mobility relative to those of standard phospholipids. The amount of each labeled phospholipid was determined by liquid scintil-

FIG. 3. Immunoblots of phospholipid biosynthetic enzymes during growth. *S. cerevisiae ade5* was grown in complete synthetic medium. Cells were harvested at the indicated time intervals, cell extracts were prepared, and 50- μ g samples were subjected to immunoblot analysis as described in the text. The figure shows portions of immunoblots showing the subunit ($M_r = 62,000$) of inositol-1-phosphate synthase (IPS), the subunit ($M_r = 34,000$) of PI synthase (PIS), and the subunit ($M_r = 23,000$) of PS synthase (PSS).

lation counting of the corresponding spots on the chromatogram. The phospholipid composition of cells was also analyzed by high-performance liquid chromatography. Cells (ca. 0.5 to 0.7 g of cell [wet weight]) were washed with distilled water and suspended in 1 ml of methanol. The cell suspension was mixed with glass beads (1 g) and disrupted with a Mini-Bead Beater (Biospec). Lipids were extracted (4) from the broken cell suspension and analyzed with a silica column (μ -Porasil; Waters Associates, Inc., Milford, Mass.) as described previously (31). This method does not separate PS from PA (31); however, the values that we obtained for PC, PE, and PI by this method were in general agreement with that obtained by the $^{32}\text{P}_i$ labeling analysis.

RESULTS

Effect of the growth phase on phospholipid biosynthetic enzyme activities in cells grown in complete synthetic medium. The effect of the growth phase on the activity of several phospholipid biosynthetic enzymes was examined. Maximum CDP-DG synthase, PS synthase, and PNMT activities were found in cells that were grown in complete synthetic medium during the exponential phase of growth (Fig. 2B). As the cells entered the stationary phase, the specific activities of CDP-DG synthase, PS synthase, and the PNMTs decreased approximately 2.5-, 4-, and 5-fold, respectively, compared with the activities of exponential phase cells (Fig. 2B). On the other hand, the specific activity of PI synthase remained fairly constant throughout growth (Fig. 2B).

To examine if the reduction in enzyme activities in the stationary phase of growth was due to a decrease in enzyme levels, the subunit levels of PS synthase, PI synthase, and inositol-1-phosphate synthase were analyzed by immunoblotting. The subunit levels of all three enzymes did not change as cells entered the stationary phase of growth (Fig. 3). The subunit levels of PS synthase and inositol-1-phosphate synthase were slightly decreased at the 72 h time point (Fig. 3). The subunit levels of CDP-DG synthase and the PNMTs were not analyzed because antibodies to these enzymes are not yet available.

Effect of the growth phase on phospholipid biosynthetic enzyme activities in cells supplemented with inositol-choline. We examined the effect of the growth phase on phospholipid biosynthetic enzymes in cells grown in complete synthetic medium supplemented with inositol-choline. The specific

activities of CDP-DG synthase, PS synthase, and the PNMTs were reduced during the exponential and stationary phases of growth (Fig. 2D). The reduced levels of CDP-DG synthase and PS synthase activities in cells grown in the presence of inositol-choline (Fig. 2D) were essentially the same as the levels of CDP-DG synthase and PS synthase activities of cells in the stationary phase when grown in the absence of inositol-choline (Fig. 2B). The PNMT activities were reduced in the exponential phase, followed by a further reduction of activity as the cells entered the early stationary phase (Fig. 2D). In the late stationary phase, the PNMT activities returned to the reduced activity levels of the exponential-phase cells grown in the presence of inositol-choline. The specific activity of PI synthase did not change significantly in cells grown in the presence of inositol-choline and remained fairly constant throughout growth (Fig. 2D).

As reported previously, when cells were grown in medium supplemented with inositol-choline, the subunit levels of PS synthase (30) and inositol-1-phosphate synthase (26) were repressed in the exponential phase of growth (Fig. 4). Thus, the decrease in enzyme activities for at least PS synthase and inositol-1-phosphate synthase (12) in cells grown in the presence of inositol-choline was due to an enzyme repression mechanism. As the cells entered the stationary phase of growth, however, the subunits levels of PS synthase and inositol-1-phosphate synthase were derepressed (Fig. 4). The derepression of inositol-1-phosphate synthase and the PS synthase subunits may be due to a depletion of inositol in the growth medium. It is known that the absence of inositol in the growth medium leads to a derepression of inositol-1-phosphate synthase (12, 13) and PS synthase (26, 30). The decrease in PI content of stationary-phase cells as compared with exponential-phase cells (see below) also suggests a depletion of inositol in the growth medium. In the absence of inositol, choline does not affect the expression of PS synthase (26, 30) or inositol-1-phosphate synthase activities (12, 13, 26). PI synthase subunit levels were constitutive throughout the exponential and stationary phases of growth when cells were supplemented with inositol-choline (Fig. 4). The growth of strain *ade5* was not significantly affected by the presence of inositol-choline in the growth medium (Fig. 2A and C).

Effect of mixing cell extracts of exponential-phase and

FIG. 4. Immunoblots of phospholipid biosynthetic enzymes during growth in medium supplemented with inositol-choline. *S. cerevisiae ade5* was grown in complete synthetic medium containing inositol (50 μM) and choline (1 mM). Cells were harvested at the indicated time intervals, cell extracts were prepared, and 50- μ g samples were subjected to immunoblot analysis as described in the text. The figure is portions of immunoblots showing the subunit ($M_r = 62,000$) of inositol-1-phosphate synthase (IPS), the subunit ($M_r = 34,000$) of PI synthase (PIS), and the subunit ($M_r = 23,000$) of PS synthase (PSS).

TABLE 1. Effect of mixing cell extracts from exponential- and stationary-phase cells on phospholipid biosynthetic enzyme activities^a

Growth condition	Sp act (nmol/min per mg) of:			
	CDP-DG synthase	PS synthase	PNMTs	PI synthase
Complete synthetic				
A. Exponential	0.51	0.42	0.53	0.31
B. Stationary	0.18	0.10	0.15	0.28
Complete synthetic-inositol-choline				
C. Exponential	0.12	0.14	0.24	0.28
D. Stationary	0.11	0.10	0.10	0.30
Mixed extracts (1:1; vol/vol)				
A + B	0.32	0.25	0.34	0.27
C + D	0.11	0.12	0.19	0.26
A + C	0.36	0.28	0.39	0.28
B + D	0.16	0.11	0.14	0.29

^a Cells were grown in complete synthetic medium in the absence and presence of inositol (50 μ M)-choline (1 mM). Cells were harvested in the exponential and stationary phases of growth, and cell extracts were prepared as described in the text. Individual cell extracts and the indicated combinations of cell extracts were assayed for the indicated enzyme activities.

stationary-phase cells on phospholipid biosynthetic enzyme activities. We examined if the reduction of enzyme activities observed in the stationary-phase cells grown in complete synthetic medium was due to the presence of a soluble inhibitor or the loss of a soluble activator. Cell extracts were prepared from exponential-phase and stationary-phase cells, the extracts were mixed and incubated for 20 min, and the activities of each enzyme were measured. The activities of the enzymes from the cell extract mixtures were the average of the specific activities of the enzymes assayed in each extract separately (Table 1). This indicates that the decrease in activities in the stationary-phase cell extracts was not due to a soluble effector molecule. When cell extracts of exponential-phase cells grown in the absence and the presence of inositol-choline were mixed and assayed for enzyme activities, the expected average activities were obtained (Table 1). This suggests that the reduction of CDP-DG synthase, PS synthase, and the PNMTs by inositol-choline was not due to the presence of soluble effector molecules.

Phospholipid composition at exponential and stationary phases of growth. Although it is known that phospholipid synthesis decreases in the stationary phase of growth (23, 33), it is not known what effect the growth phase has on the phospholipid composition of the cell. The composition of the major phospholipids of cells grown in complete synthetic medium in the absence and presence of inositol-choline is presented in Table 2. The phospholipid composition of *ade5* cells in the exponential phase of growth in complete synthetic medium was similar to that reported by Klig et al. (26) and was in general agreement with that reported previously (16, 18). In the stationary phase, the cellular content of PC, PE, PI, and PMME increased whereas that of PS, PA, and PDME decreased.

The addition of inositol-choline to the growth medium resulted in a decrease in PC, PE, PS, and PDME and an increase in PI and PMME in exponential-phase cells as compared with exponential-phase cells grown without supplementation. Greenberg et al. (16) and Klig et al. (26) have shown similar changes in phospholipid composition as a

result of growth with inositol-choline; however, these investigators showed a more dramatic decrease in the PS content. This difference could be due to the cell number used for phospholipid analysis and the method of phospholipid extraction. The addition of inositol-choline to complete synthetic medium resulted in a twofold increase in the PI to PS ratio of cells in the exponential phase as compared with exponential-phase cells grown without supplementation. This was mainly due to an increase in the PI content of cells, which was presumably due to an increase in PI synthesis, given the readily available exogenous inositol (15). In stationary-phase cells grown with inositol-choline, PC, PE, and PMME increased, whereas PI, PS, PDME, and PA decreased compared with exponential-phase cells grown with inositol-choline.

DISCUSSION

As *S. cerevisiae* cells enter the stationary phase of growth, triacylglycerols accumulate at the expense of total phospholipid synthesis (23, 33). In this study we have shown that the activities of the phospholipid biosynthetic enzymes in the primary route of biosynthesis from PA to PC (CDP-DG synthase, PS synthase, and the PNMTs) decreased in the stationary phase of cells grown in medium in the absence (derepressed condition) or presence (repressed condition) of inositol-choline. This decrease in enzyme activities was not due to the presence of soluble effector molecules in cell extracts, as indicated by the mixing experiments. However, these experiments do not rule out the presence of membrane-bound effectors which could have modulated the enzyme activities. We were not able to establish any regulation of the phospholipid biosynthetic enzyme activities in cell extracts under assay conditions that favor phosphorylation or dephosphorylation reactions (data not shown). The reduction in PS synthase activity in the stationary phase of derepressed or repressed cells was not due to a decrease in the PS synthase subunit in cell extracts. This suggests that the reduction in activity for at least PS synthase is not due to a reduction in enzyme synthesis or enzyme turnover. We also examined the effect of sterols on PS synthase activity because sterol synthesis increases in the stationary phase of

TABLE 2. Phospholipid composition of cells grown in the absence and presence of inositol-choline^a

Growth condition	% of total phospholipid of:							
	PC	PE	PI	PS	PMME	PDME	PA	Other
Complete synthetic								
Exponential	38.0	24.7	13.9	11.0	2.0	1.6	1.4	7.4
Stationary	40.0	26.9	18.0	7.4	2.9	0.4	1.0	3.4
Complete synthetic-inositol-choline								
Exponential	35.1	21.9	26.0	10.0	2.2	0.7	1.4	2.7
Stationary	38.0	25.2	20.9	7.1	3.3	0.6	0.7	4.2

^a Cells were grown in complete synthetic medium in the absence and presence of inositol (50 μ M)-choline (1 mM). Cells were harvested in exponential (3.6×10^7 cells per ml) and stationary (1.2×10^8 to 1.7×10^8 cells per ml) phases of growth. The phospholipid composition was determined by ³²P labeling and high-performance liquid chromatography, as described in the text. The data presented, with the exception of values for PS, PA, PMME, and PDME, are the averages of both analyses. Other includes the pooled percentages of minor phospholipid species.

growth (23). Ergosterol (1 mM) or cholesterol (1 mM) did not affect the activity of purified PS synthase assayed in the presence of Triton X-100 (data not shown).

The phospholipid composition of cells changed in the stationary phase as compared with the exponential phase of growth. Changes in the composition of the major phospholipids or, possibly, the minor phospholipid components could be one of the mechanisms by which cells regulate the phospholipid biosynthetic enzymes. It is worth noting that the PI to PS ratio increased about twofold in the stationary phase of growth compared with the exponential phase of growth in medium without inositol-choline. Similarly, the PI to PS ratio increased about twofold in exponential-phase cells grown in medium containing inositol-choline compared with exponential-phase cells grown without supplementation. This PI to PS ratio was conserved in stationary-phase cells grown in medium containing inositol plus choline, whereas the content of the remaining phospholipids were similar to that of stationary-phase cells grown without inositol-choline. The PC to PE ratio did not change significantly under the growth conditions used in this study. Results of studies with purified yeast PS synthase reconstituted into unilamellar PC-PE-PI-PS vesicles have shown that increases in the PI to PS ratio in vesicles cause a two- to threefold decrease in enzyme activity (23a). Changes in the PC to PE ratio do not affect reconstituted activity in PC-PE-PI-PS vesicles (23a). It is possible that the alterations in the PI to PS ratio of stationary-phase cells compared with exponential-phase cells may be responsible for the reduction in PS synthase activity of stationary-phase cells. This correlation, however, may be an oversimplification of the regulation that occurs in the stationary phase. The reconstituted enzyme system did not contain minor membrane components that may be involved in enzyme regulation and were present in the cell extracts used in this study. Furthermore, the activity of PS synthase in this study was measured with a Triton X-100-mixed micelle substrate, whereas the activity of reconstituted PS synthase was previously measured in the absence of Triton X-100 (23a).

Unlike the enzymes involved in the primary route for PC biosynthesis, PI synthase is constitutive under all the growth conditions studied thus far (15, 26). If triacylglycerol synthesis is increased in the stationary phase of growth as a result of induced PA phosphatase activity (23), then the pool of CDP-DG for phospholipid biosynthesis would decrease. This available pool of CDP-DG must be partitioned between the synthesis of PI and PS. Because PI is an essential membrane phospholipid that is needed for various cellular processes (3, 19), continued synthesis of PI from the available pool of CDP-DG would be an important reaction for the cell to maintain throughout its growth. PS synthase activity was reduced in stationary-phase cells, whereas PI synthase was constitutive, thus favoring PI synthesis. Results of this and previous studies (26, 30) also suggest that the competition of PI synthase and PS synthase for CDP-DG is regulated by the repression of PS synthase in response to water-soluble phospholipid precursors. In the stationary phase, however, PS synthase activity decreased without a corresponding decrease in enzyme subunit levels. Therefore, the overall coordinate regulation between PC biosynthesis (via PS) and inositol biosynthesis involves the availability of water-soluble phospholipid precursors and the growth phase of the culture. Changes in the growth phase of the culture result in altered patterns of phospholipid composition that may be involved in the overall regulation of enzyme activities.

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