The Role of Phosphatidylcholine Biosynthesis in the Regulation of the INO1 Gene of Yeast

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In yeast, as in other eukaryotes, phosphatidylcholine (PC) can be synthesized via methylation of phosphatidylethanolamine or from free choline via the CDP-choline pathway. In yeast, PC biosynthesis is required for the repression of the phospholipid biosynthetic genes, including the INO1 gene, in response to inositol. In this study, we analyzed the effect of mutations in genes encoding enzymes involved in PC biosynthesis on the transcriptional regulation of phospholipid biosynthetic genes. We report that repression of INO1 transcription in response to inositol is clearly dependent on ongoing PC biosynthesis, but it is independent of the route of synthesis. Our results also suggest that intermediates in the phosphatidylethanolamine methylation and CDP-choline pathways are not responsible for generating the regulatory signal that results in repression of INO1 and other coregulated genes of phospholipid biosynthesis. Furthermore, repression of INO1 is not tightly correlated to the proportion of PC in the total cellular phospholipids. Rather, we report that when the rate of synthesis of PC becomes growth limiting, the addition of inositol fails to repress the phospholipid biosynthetic genes, but when the rate of PC synthesis is sufficient to sustain normal growth, the addition of inositol to the growth medium has the effect of repressing INO1 and other phospholipid biosynthetic genes.

Phosphatidylcholine is synthesized in eukaryotic cells via two distinct pathways. One pathway involves three sequential methylations of phosphatidylethanolamine (PE) (1). Alternatively, PC can be synthesized from free choline via the CDP-choline (“Kennedy”) pathway (2). These two pathways are found in all eukaryotes that have been investigated, including mammals (3) and yeast (4, 5). In mammals, synthesis from free choline via the CDP-choline pathway represents the major route of PC biosynthesis. Synthesis of PC via the methylation of PE has been detected only in hepatocytes and brain cells (3). In yeast, PC can be synthesized de novo via the methylation pathway or, when choline is present in the growth medium, via the CDP-choline pathway (Fig. 1).

Phospholipid biosynthesis is highly regulated in yeast and, curiously, the regulation of inositol- and choline-containing phospholipids is coordinated. Much of this coordinate regulation occurs at the level of gene transcription in response to the soluble precursors inositol and choline. In the presence of inositol, transcription of coregulated biosynthetic genes is repressed. If choline is added to medium in which inositol is already present, the genes are further repressed. However, if choline is present in the growth medium by itself, it has little or no effect on transcription of the coregulated genes. Genes that have been shown to exhibit this pattern of transcriptional regulation include INO1 (inositol-1-phosphate synthase), CHO1 (choline kinase), CPT1 (choline phosphate-transferase), CHO2 (cholinephosphotyidylinositol-phosphate synthase), CHO2/PEM1 (phosphatidylethanolamine methyltransferase), and OPI3/PEM2 (phospholipid methyltransferase) (Fig. 1). The genes encoding the inositol and choline transporters are also subject to this regulation (6–8). Furthermore, the structural genes that show this coordinated regulation in response to inositol and choline all respond to a single set of regulatory genes, including INO2, INO4, and OPI1 (6–8).

Another intriguing aspect of this coordinated regulatory response is its dependence on ongoing PC biosynthesis. Yeast strains carrying mutations in the CHO1, CHO2, or OPI3 structural genes are conditionally defective in PC biosynthesis (see Fig. 1 for the position of each lesion in the pathway). The cho1, cho2, and opi3 mutants also exhibit a conditional overproduction of inositol (Opi phenotype) (9–14). The Opi phenotype is indicative of overexpression of inositol-1-phosphate synthase due to misregulation of the INO1 gene (10, 12). The cho1, cho2, and opi3 mutants all have been shown to derepress INO1 in the presence of inositol (9–11, 13, 14). The other coregulated enzymes, including the CHO1, CHO2, and OPI3 gene products themselves, as well as enzymes of the CDP-choline pathway, show this same pattern of aberrant regulation when PC biosynthesis is interrupted (6). Normal regulation in response to inositol is restored in these mutants and their Opi phenotype is eliminated if a metabolite that enters the PC biosynthetic pathway downstream of the genetic block is supplied exogenously. Thus, INO1 regulation is restored in cho1 mutants (which are defective in phosphatidylserine biosynthesis) if ethanolamine, monomethylethanolamine, dimethylethanolamine, or choline is supplied (13). Regulation of INO1 is restored in cho2 mutants (which are defective in PE methylation) in response to each of the three methylated species, but not in response to ethanolamine (11). In opi3 mutants, regulation is restored only in response to dimethylethanolamine or choline (14). However, the effect of mutations in the CDP-choline path-
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Fig. 1. Phospholipid metabolic pathways in yeast. These pathways and the gene–enzyme relationships involved have recently been reviewed (6). PA, phosphatic acid; DAG, sn-1,2-diacylglycerol; CDP-DG, cytidine diphosphate diacylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PM, phosphatidylmonomethylethanolamine; PD, phosphatidyl(dimethylethanolamine); C-P, choline phosphate; CDP-C, cytidine diphosphate choline; E-P, ethanolamine phosphate; CDP-E, cytidine diphosphate ethanolamine; PS1, PI synthase; PS2 and PS2, PS decarboxylases.

way for PC biosynthesis on this regulation had not been explored when we commenced this study.

In the present study, we have analyzed the role of PC metabolism in regulation of phospholipid biosynthesis by determining the effects of mutations in both routes of PC biosynthesis, alone or in combination. Our results demonstrate that the regulatory response is independent of the route of synthesis of PC. Analysis of the phospholipid content and growth properties of strains carrying mutations in PC biosynthesis suggests that the transcriptional response to inositol is most likely generated by the formation of PC itself and not by the availability of choline or any other intermediate in PC biosynthesis.

EXPERIMENTAL PROCEDURES

Materials—Sources of materials were: [32P]orthophosphate (carrier free), [3H]choline chloride (specific activity, 200 Ci/mmol), [methyl-14C]choline chloride (activity, 40–60 mCi/mmol), DuPont NEN; SG81 paper, GF/A glass fiber filters, Whatman; nitrocellulose, Schleicher & Schuell; SP6/T7 Transcription Kit, Boehringer Mannheim. All other materials were reagent grade or better.

Construction of Strains Defective in PC Biosynthesis—To analyze which steps in PC biosynthesis are required for generation of the signal necessary for proper regulation of the coregulated genes involved in phospholipid biosynthesis, we constructed strains with defects in either one or both routes of PC biosynthesis (i.e., CDP-choline and methylation pathways, as shown in Fig. 1). To block the methylation pathway, we chose the cho2 mutant, which is defective in the first methylation step in the conversion of PE to phosphatidylinomonomethylethanolamine. Strains carrying the cho2 gene disruption have grossly altered phospholipid composition (PC content, between 6 and 10% of total phospholipid) and grow considerably slower than wild-type strains in the absence of choline or its methylated precursors (11, 15).

To block the CDP-choline pathway, we used a strain in which the CKI1 gene was disrupted, as well as a double mutant strain carrying CPT1 and EPT1 gene disruptions simultaneously. The CKI1 gene encodes choline kinase, and when this gene is disrupted, yeast cells lose almost all of their choline kinase activity and most of their ethanolamine kinase activity (16). The cpt1 and ept1 mutants block the last steps of the CDP-choline pathway and the CDP-ethanolamine pathway, respectively, which are necessary for the formation of PC and PE from sn-1,2-diacylglycerol and CDP-choline or CDP-ethanolamine. We used a double mutant strain since it has been shown that the CPT1 and EPT1 gene products are both capable of catalyzing the cholinephosphotransferase reaction in vitro (17). The contribution of the EPT1 gene product to this reaction is much lower than that of the CPT1 gene product, but it is still noticeable in vivo (18).

The HJ000 strains (cpt1, ept1) and their congenic wild-type parental strain, DBY746, were generously provided by Dr. Robert Bell (Duke University Medical Center). To construct a CHO2 disruption in the genetic background of cpt1, ept1, the SulI-BgII internal fragment of the CHO2 gene (11, 15), carried on plasmid pSPT18 (Boehringer Mannheim), was replaced with the TRP1 gene. This construct was used to generate a disruption of the CHO2 gene in HJ000 (cpt1, ept1) and its congenic wild-type parental strain, DBY746, by homologous recombination (19). Stable TRP+ transformants were tested for the characteristic cho2 mutant inositol excretory (Opi) phenotype (11, 12), and the disruption of a CHO2 gene was confirmed by the absence of CHO2 mRNA on Northern blots. In this way, we were able to construct strains with blocks in the methylation pathway, the CDP-choline pathway, or simultaneously in both pathways. The full genotypes of these strains are given in Table I.

Strain CTY393 (chi1 gene disruption mutant) was provided by Dr. Vytais Bankaitis (University of Alabama at Birmingham). Strain CTY393 was crossed to w303-1B and sporulated. Spore HJ335 was crossed to DC5 to generate diploid SH336. A CHO2 disruption in the chi1 background was carried out in diploid strain SH336. The CHO2 gene in SH336 was disrupted by LEU2 gene using a construct similar to one used by Summers et al. (11, 15). Integration of the LEU2 gene in the CHO2 locus was confirmed by PCR. Strain SH336, with a disrupted CHO2 gene, was sporulated and sister spores 1-A, 1-B, 1-C, and 1-D (genotypes given in Table I) were used in further studies.

Culture Conditions—Yeast strains were maintained on YEPD medium (1% yeast extract, 2% Bactopeptone, 3% glucose). Vitamin-defined synthetic media contained, per liter: 30 g of glucose, 5 g of ammonium sulfate, 1 g of potassium phosphate monobasic, 0.5 g of manganese sulfate, 0.1 g of calcium chloride, 0.2 mg of ferric chloride, 0.04 mg of cupric sulfate, 0.1 mg of potassium iodide, 0.2 mg of potassium phosphate monobasic, 0.5 g of magnesium sulfate, 1 g of sodium chloride, 0.1 g of calcium chloride, 0.5 mg of boric acid, 0.04 mg of cupric sulfate, 0.1 mg of potassium iodide, 0.2 mg of ferric chloride, 0.4 mg of manganese sulfate, 0.2 mg of sodium molybdate, 0.4 mg of zinc sulfate, 20 mg of adenine, 20 mg of arginine, 20 mg of histidine, 60 mg of leucine, 230 mg of lysine, 20 mg of methionine, 300 mg of threonine, 20 mg of tryptophan, 40 mg of uracil, 2 μg of biotin, 400 μg of panthothenate, 2 μg of folic acid, 400 μg of niacin, 200 μg of p-amino benzoic acid, 400 μg of pyridoxine hydrochloride. Where indicated, media were supplemented with 75 μg inositol (1') and/or 1 μm choline (C'). All cultures were grown aerobically at 30°C with shaking.

Yeast Genetic Manipulations—Genetic techniques such as mating, sporulation, and tetrad dissection were carried out using standard methodologies (20). Yeast transformation was done by the lithium acetate method (21) with minor modifications.

RNA Analyses—RNA probes for Northern blot hybridization were synthesized according to manufacturer recommendations for the SP6/T7 Transcription Kit (Boehringer Mannheim) from plasmids described in Hudak et al. (22), linearized with a restriction enzyme, and transcribed with a RNA polymerase as follows (plasmid/restriction enzyme/RNA polymerase): pAB309/EcoRI/SP6 (TCM1), pMT209/EcoRI/SP6 (OPI3), pH310/HindIII/T7 (INO1), pTG109/BamHI/T7 (CHO2). RNA was isolated from yeast using glass bead disruption and hot phenol extraction (23). The TCM1 ribosomal protein gene, expression of which is unaffected by availability of inositol and choline, was used as a standard for RNA loading, as described previously (10). Northern hybridization was performed essentially as described by Hirsch and Henry (10), and the results were visualized by autoradiography and quantified by an AMBIS 4000 phosphorimager (AMBIS, Inc.).

Phospholipid Composition—Steady-state labeling with [3H]phosphatidylethanolamine was performed following the method of Atkinson et al. (24). Cells were labeled for at least five generations with 5 μCi of [3H]phosphatidylethanolamine/nl in synthetic media, as described above, and harvested in the late logarithmic phase of growth (unless otherwise indicated). Labeled lipids were extracted as described by Atkinson et al. (24). Two-dimensional paper chromatography on silic acid-impregnated paper was carried out using the method of Stein and Lester (4). Labeled spots corresponding to specific lipids were quantified using an AMBIS 4000 phosphorimager.

Incorporation of [methyl-14C]Choline into PC—Yeast cells were
TABLE I  
Yeast strains

| Strain   | Genotype                                                                 | Source         |
|----------|---------------------------------------------------------------------------|----------------|
| DBY746   | Mata his3-D1, leu2-3,112, ura3-52, trp1-289                               | R. Bell        |
| DBY746 cho2 | Mata his3-D1, leu2-3,112, ura3-52, trp1-289, cho2 : TRP1                     | This study     |
| HJ000    | Mata his3-D1, leu2-3,112, ura3-52, trp1-289, cpt1 : LEU2, ept1-1 : URA3 | R. Bell        |
| HJ000 cho2 | Mata his3-D1, leu2-3,112, ura3-52, trp1-289, cpt1 : LEU2, ept1-1 : URA3, cho2 : TRP1 | This study   |
| CTY393   | Mata his3-200, ura3-52, lys2-280, 1-91 : HIS3                              | V. Bankaitis   |
| w303-1B  | Mata ade2-1, can1-100, his3-115, leu2-3,112, trp1-1, ura3-52               | R. Rothstein   |
| DC5      | Mata his3-11,15, leu2-3,112                                              | J. Broach      |
| SH335    | Mata leu2-3,112, ade2, ura3, cin1 : HIS3                                   | This study      |
| SH336 (diploid) | Mata/Mata cin1 : HIS3/CHR1, CHO2/CHO2, his3/3his3, leu2/2leu2, ade2/ADE2, ura3/URA3 | This study  |
| 1-9A     | Mata ade2, ura3, his3, leu2                                                | This study      |
| 1-9B     | Mata ade2, leu2, his3, cin1 : HIS3                                         | This study      |
| 1-9C     | Mata leu2, his3, cin1 : HIS3, cho2 : LEU2                                   | This study      |
| 1-9D     | Mata ura3, leu2, his3, cho2 : LEU2                                         | This study      |

grown for at least five to six generations with 0.2 μCi/ml of [methyl-14C]choline chloride (specific activity, 40–60 mCi/mmol) in vitamin-defined synthetic media containing 1 μg of unlabeled choline. Cultures were harvested in late logarithmic phase to early stationary phase of growth. Lipids were extracted as described by Atkinson et al. (24) and separated in the first dimension using the method of Steiner and Lester (4). The position of radioactively labeled PC was determined by autoradiography. Labeled spots were removed and counted by liquid scintillation.

**Choline Uptake—**Yeast cultures were grown overnight in vitamin-defined synthetic medium containing 75 μM inositol and 1 mM choline (1°C) to mid-logarithmic phase of growth, when radiolabeled choline was added (1 μCi/ml, 40–60 mCi/mmol [methyl-14C]choline chloride). Uptake was allowed to proceed for 60 min at 30°C with shaking. Samples were taken at indicated time points, and choline uptake was terminated by vacuum filtration of cultures through Whatman GF/A glass fiber filters, which were immediately rinsed with 15 ml of ice-cold 20 mM choline. The filters were allowed to dry, and the associated radioactivity was determined by liquid scintillation counting.

**RESULTS**

**Phenotypes of the Strains Defective in PC Biosynthesis—**The triple mutant strain cpt1, ept1, cho2 (HJ000 cho2) is viable but displayed relatively slow growth regardless of the presence or absence of choline in the medium. The doubling time for this strain was 6.5 h in synthetic medium with or without choline (Table II). Summers et al. (11) reported that when cho2 strains are shifted to choline-free medium, they initially grow at a rate comparable to their rate of growth in choline-supplemented medium (doubling time, 2.5–3.0 h). However, after five to six generations in the absence of choline, the doubling time of cho2 strains slows to 6 h or longer. Thus, the growth rate we observed for the cho2, cpt1, ept1 strain was similar to the reported sustainable growth rate (11) for cho2 strains grown for an extended period of time in the absence of choline. The doubling time of the cho2 strain, DBY746 cho2, in YEPD medium was comparable to the congenic DBY746 wild-type strain (1.5 h). However, the growth rate of the cpt1, ept1, cho2 triple mutant (which is congenic to DBY746 and DBY746 cho2) in YEPD medium was 6.5 h, comparable to its rate of growth in synthetic medium. Moreover, regardless of growth condition, the cells of the triple mutant displayed morphological abnormalities not seen in the DBY746 or DBY746 cho2 strains, including large, elongated cells with multiple buds and a tendency to clump. The triple mutant also generates respiratory-deficient petites at high frequency (25); a characteristic not observed in the wild type, the cho2, or the cpt1, ept1 congenic strains. In the absence of choline, the cho2, cin1 strain (1-9C) exhibited a doubling time of 6.5 h, comparable to other cho2 strains and the triple mutant, cpt1, ept1, cho2. However, unlike the cpt1, ept1, cho2 strain, the cho2, cin1 strain doubled in a time-supplemented with choline (Table II), a doubling time comparable to cho2 single mutants growing in the presence of choline.

The overproduction of inositol (Opi) phenotype (12) is indicative of overexpression of inositol-1-phosphate synthase due to misregulation of the ILO1 gene. Neither of the wild-type strains used in this study (DBY746 and 1-9A) or the strains carrying mutations in the CDP-choline pathway (HJ000 and 1-9B) displayed an Opi phenotype, regardless of the presence or absence of choline in the medium. However, the cho2 single mutant strains used in this study (DBY746 cho2 and 1-9D) displayed the Opi phenotype, but only on media lacking choline, consistent with the previously reported phenotype of cho2 strains (11). Those strains with metabolic lesions in both pathways leading to synthesis of PC, i.e. cpt1, ept1, cho2 (HJ000 cho2) and cho2, cin1 (1-9C), displayed an Opi phenotype regardless of the presence or absence of choline in the medium. However, the size of the Opi inositol excretion ring was considerably larger for the cpt1, ept1, cho2 strain (HJ000 cho2) compared to the cho2, cin1 strain (1-9C).

**Choline Uptake—**Mutants with defects in the CDP-choline pathway display lower levels of choline uptake than wild-type cells, but the decreased rate of choline import is apparent only after metabolites of the CDP-choline pathway have accumulated within the cell (18). We assessed levels of choline uptake in the cpt1, ept1, cho2 triple mutant, in the cpt1, ept1 double mutant, and in the cho2 mutant and compared them to the congenic wild-type strain, DBY746 (Fig. 2). The rate of choline uptake in the strains containing the cpt1 and ept1 mutations, cpt1, ept1 (HJ000) and cpt1, ept1, cho2 (HJ000 cho2), was decreased by about one-half compared to their CPT1, EPT1 counterparts (wild-type (DBY746) or DBY746 cho2 strains, respectively). In contrast, strains carrying the cho2 mutation in the CPT1, EPT1 background (DBY746 cho2) or in combination with the cpt1 and ept1 mutations (HJ001 cho2) had significantly increased uptake of choline when compared to their CHO2 counterparts (i.e. the wild-type strain, DBY746, or the cpt1, ept1 strain, HJ000, respectively; Fig. 2).

**Phospholipid Analysis—**To determine whether the cin1 mutation or the combination of cpt1 and ept1 mutations could completely prevent synthesis of PC from 13C-labeled choline, mutant strains were grown in defined medium containing 75 μM inositol and 1 mM choline in the presence of 13C-labeled choline and the lipids were then extracted. Incorporation of the label into PC was analyzed as described under “Experimental Procedures.” Incorporation of labeled choline into PC in the cin1 strain (1-9B) occurred at approximately 10% of the level of incorporation observed in the wild-type strain (data not shown). This result is consistent with previous reports that the cin1 mutant retains some limited ability to incorporate choline into PC via the CDP-choline pathway (16, 25). In the cho2, cin1 (1-9C) strain, incorporation of labeled choline into PC was elevated about 5-fold compared to the congenic cin1 strain and was approximately 50% of the level observed in wild-type cells (data not shown), as previously reported (25). Strains carrying
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TABLE II

| Strain       | Genotype                        | Medium                          | Proportion of phospholipids (%) |
|--------------|---------------------------------|---------------------------------|---------------------------------|
|              |                                 |                                 | h                               |
| DBY746       | Wild type I                     | I'C'                            | 2.0                             |
| DBY746       | Wild type I                     | I'C'                            | 2.0                             |
| DBY746       | cho2                            | I'C'                            | 2.5                             |
| DBY746       | cho2                            | I'C'                            | 6.0                             |
| HJ000        | cpt1, ept1                      | I'C'                            | 2.0                             |
| HJ000        | cpt1, ept1                      | I'C'                            | 2.0                             |
| HJ000        | cho2, cpt1, ept1, cho2           | I'C'                            | 6.5                             |
| HJ000        | cho2, cpt1, cho2                | I'C'                            | 6.5                             |
| 1-9D         | cho2                            | I'C'                            | 3.0                             |
| 1-9D         | cho2                            | I'C', stationary phase           | 0.8                             |
| 1-9C         | cho2, chi1                       | I'C'                            | 2.7                             |
| 1-9C         | cho2, chi1                       | I'C', stationary phase           | 0.1                             |

* ND, not detected.

Fig. 2. Choline uptake. Wild-type (DBY746, ▲, cho2 (DBY746, ●), cpt1, ept1 (HJ000, □), and cpt1, ept1, cho2 (HJ000, ▣) strains were grown in vitamin-defined medium containing 75 μM inositol and 1 μM choline to mid-logarithmic phase of growth, at which point radiolabeled [methyl-14C]choline was added. At the indicated time points, samples were collected, cells were washed on Whatman GF/A glass fiber filters, and radioactivity associated with cells was determined by liquid scintillation counting, as described under "Experimental Procedures." Values were normalized to the turbidity of culture (A595) at the time of sampling.

a combination of the cpt1 and ept1 mutations (i.e. cpt1, ept1 (HJ000) or cpt1, ept1, cho2 (HJ000 cho2)) were both incapable of incorporating any detectable labeled choline into PC (data not shown), confirming that the block in PC biosynthesis via the CDP choline pathway is complete in the cpt1, ept1 double mutant, as reported previously (17, 18).

Phospholipid composition was determined for the various strains grown in media containing 75 μM inositol, with or without choline (Table II). Cells were labeled to steady-state with 32P, and lipids were extracted and analyzed as described under "Experimental Procedures." The cho2 strains used in this study (DBY746 cho2 and 1-9D), when grown in the presence of choline, contained levels of PC comparable to the comparable wild-type strains. When cho2 strain, DBY746 cho2, was grown in the absence of choline, however, the proportion of PC in its total phospholipid dropped dramatically and the level of PE rose in comparison to the wild-type strain (DBY746) grown under identical conditions. These data are in agreement with previously published phospholipid compositions for cho2 mutant strains (11). The phospholipid composition of the cpt1, ept1 HJ000 strain resembled the composition of the congenic wild-type (DBY746) strain. The triple mutant cpt1, ept1, cho2 (HJ000 cho2), which is blocked in both pathways leading to PC biosynthesis, exhibited a phospholipid profile that did not change in response to the presence or absence of choline in the growth medium and resembled that of the congenic cho2 strain (DBY746 cho2) grown in the absence of choline (Table II).

Surprisingly, despite its increased growth rate in response to choline and its residual ability to incorporate choline into PC, as described above, the cho2, chi1 double mutant (1-9C), grown in the presence of choline and harvested during logarithmic growth, contained a low level of PC. The proportion of PC in the cho2, chi1 strain was comparable to the level of PC observed in the much slower growing cpt1, ept1, cho2 strain. The proportion of PC, however, was slightly higher when cho2, chi1 cells grown in the presence of choline were harvested in stationary phase compared to logarithmic phase (Table II).

Effect of Mutations in PC Biosynthesis on INO1 and OPI3 Expression—Expression of the INO1 gene in exponentially growing cell cultures was assessed by Northern blot analysis, using the congenic set of strains: wild-type (DBY746); cho2 (DBY746 cho2); cpt1, ept1 (HJ000); and cho2, cpt1, ept1 (HJ000 cho2). A Northern blot illustrating the expression of the INO1 transcript in each of the four strains is depicted in Fig. 3A, and quantitation of the INO1 mRNA levels in this experiment are given in Fig. 3B. As reported previously (10), INO1 expression in the wild-type cell strain was maximally repressed by the addition of inositol in the absence of choline, whereas the addition of choline in the absence of inositol had little effect. In agreement with results reported previously (11), inositol alone had little effect on INO1 expression in the cho2 single mutant (DBY746 cho2), whereas the addition of choline plus inositol resulted in virtually complete repression of INO1. In contrast to the pattern observed in cho2 strains, regulation of INO1 expression in the cpt1, ept1 strain was indistinguishable from that observed in the wild-type strain. The triple mutant strain cpt1, ept1, cho2 (HJ000 cho2), however, showed derepressed levels of INO1 in all four growth media. In other words, the triple mutant failed to repress INO1 in response to any combination of inositol plus choline.

Expression of the coregulated OPI3 (PEM2) gene encoding phospholipid N-methyltransferase (Fig. 1) was also examined in the cpt1, ept1 (HJ000) strain and

Steady-state phospholipid profiles of yeast grown in vitamin-defined synthetic media with 75 μM inositol (I') and with (C') or without (C) 1 μM choline. Yeast cultures were grown for at least five generations with [32P]orthophosphate containing 5 μCi/ml. Until otherwise stated, the yeast cultures were harvested in midexponential phase of growth. Glycerophospholipids were extracted, resolved, and quantitated as described under “Experimental Procedures.” The designation “other” includes mainly polar lipids migrating with the origin and other minor lipids, like phosphatidylglycerol, cardiolipin, and lysosphospholipids. The complete genotypes of the strains are given in Table I.
in the triple mutant cpt1, ept1, cho2 (HJ000 cho2). Inositol supplementation in the absence of choline resulted in an approximately 50–60% reduction in \( \text{OP13} \) mRNA abundance in \( \text{cpt1, ept1 cells} \), whereas the addition of choline had little effect whether inositol was present or not (Fig. 4). In the triple mutant cho2, cpt1, ept1 (HJ000 cho2), expression of the \( \text{OP13} \) gene was completely constitutive and failed to respond to any combination of inositol and choline. Thus, the \( \text{OP13} \) and \( \text{INO1} \) genes, which have both been shown previously to be regulated coordinately in response to inositol and choline (6), both failed to respond to any combination of inositol and choline in the \( \text{cpt1, ept1, cho2 triple mutant background} \).

The enzymes of phospholipid biosynthesis in yeast are regulated in response to growth phase, as well as to the presence of the soluble precursors, inositol and choline (reviewed in Ref. 6). In wild-type cells, the genes encoding these enzymes are maximally expressed in the absence of inositol and choline during logarithmic phase, but even in the absence of phospholipid precursors (i.e. derepressing growth conditions), expression of these genes decreases as the cells approach stationary phase (26). \( \text{INO1} \) regulation in response to inositol and choline was examined in the cho2, \( \text{cki1} \) double mutant and in the related cho2 strain at various stages of growth (Fig. 5). As discussed above, the strains carrying the cho2 mutation were not able to repress \( \text{INO1} \) transcription in response to inositol alone during the logarithmic phase of growth (Figs. 3 and 5). However, the \( \text{INO1} \) gene was repressed in cho2 cells upon addition of inositol and choline during logarithmic growth (Figs. 3 and 5). In the presence of inositol when no choline was present, \( \text{INO1} \) expression continued at derepressed levels in cho2 cells through early stationary phase. As the cells entered stationary phase, however, the level of transcript did decline somewhat (Fig. 5; time point 3) and by late stationary phase, \( \text{INO1} \) expression was considerably diminished (Fig. 5, time point 4).

The expression of \( \text{INO1} \) mRNA decreases in the \( \text{cpt1, ept1} \) (HJ000) strain in stationary phase even in the absence of inositol and choline (data not shown), as it does in wild-type cells (26). In the triple mutant cho2, cpt1, ept1 strain, \( \text{INO1} \) expression also declined somewhat as cells approached stationary phase (data not shown). The residual stationary phase expression of \( \text{INO1} \) in the triple mutant was comparable to the response observed in cho2-bearing strain 1-9D (Fig. 5).

Like other cho2-bearing strains, the cho2, \( \text{cki1} \) double mutant was unable to repress the \( \text{INO1} \) gene in response to inositol alone during the logarithmic phase of growth. However, unlike the \( \text{cpt1, ept1, cho2} \) strain, which is completely constitutive, the cho2, \( \text{cki1} \) strain was able to repress \( \text{INO1} \) partially, but not completely, if choline was present along with inositol during logarithmic growth (Fig. 5; compare \( \text{I}^+ \text{C}^- \) growth condition to the \( \text{I}^+ \text{C}^- \) condition, time point 1). The degree of repression of \( \text{INO1} \) in the cho2, \( \text{cki1} \) strain in response to choline plus inositol in logarithmic and early stationary phase did not appear to be quite as complete as the repression observed in the related cho2 single mutant strain under comparable conditions (Fig. 5, compare time point 1 under \( \text{I}^+ \text{C}^- \) growth conditions for strains 1-9D (cho2) and 1-9C (cho2 \( \text{cki1} \))). Moreover, in contrast to the related cho2 strain, when the cho2, \( \text{cki1} \) strain was grown in the presence of inositol plus choline (a condition that
is partially repressing for this strain during logarithmic phase (see Fig. 5, time point 1 under the 1°C growth condition). INO1 expression appeared to derepress as the cells entered late stationary phase (Fig. 5, time point 4, under the 1°C growth condition). High derepressed levels of INO1 expression were also observed in the cho2, cki1 strain in stationary phase when inositol, but no choline, was present (Fig. 5, time point 4, 1°C growth condition). The extreme stationary phase derepression of INO1 in the cho2 cki1 strain contrasts markedly with the decline in INO1 expression seen in stationary phase in all other cho2 strains under these same growth conditions, including the cpt1, ept1, cho2 triple mutant.

**DISCUSSION**

The data presented here demonstrate that repression of the INO1 gene in response to inositol is dependent upon ongoing PC biosynthesis. This regulation is, however, independent of the route of PC biosynthesis. The cpt1, ept1 strain has a complete block in PC biosynthesis via the CDP-choline pathway but retains the ability to synthesize PC via methylation of PE. This strain also shows normal regulation of the INO1 and OPI3 genes in response to inositol. The cho2 mutant strain is capable of repressing the INO1 gene in response to inositol, but only if choline is present, thereby permitting PC biosynthesis via the CDP-choline pathway. Under these circumstances, the cho2 defect in PE methylation is simply bypassed, not corrected. Thus, neither an intact CDP-choline pathway nor a fully functional PE methylation pathway is essential to the correct regulation of INO1 and other coregulated genes in response to inositol.

The cho2 mutant strains, growing in the absence of exogenous choline, fail to repress the INO1 gene in response to inositol. Thus, the reduced level of PC biosynthesis supported by the crippled PE methylation pathway in the cho2 mutant strain is insufficient to permit correct regulation of INO1, unless additional PC biosynthesis occurs via the CDP-choline pathway. Since the cpt1, ept1, cho2 strain has no residual ability to synthesize PC via the CDP-choline pathway, supplementation with choline fails to restore regulation of INO1. The cho2, cki2 strain has a partially functional CDP-choline pathway, as well as a partially functional PE methylation pathway. In this strain, partial repression of INO1 in response to inositol does occur during logarithmic phase if choline is also present (Fig. 5). However, INO1 repression under these conditions does not appear to be as complete as in a wild-type strain or in a cho2 strain grown in the presence of inositol and choline. Thus, the level of PC biosynthesis supported by the cho2, cki1 strain, even when it is supplied with choline, appears to be at the very margin of the minimum required for correct INO1 regulation during the logarithmic phase of growth.

When cultured in the absence of choline for an extended period, cho2 strains grow with a doubling time of 6 h or greater and PC content drops to 10% or less of total phospholipid (Refs. 11, 15; Table II). At this low proportion of PC, cho2 strains grow slowly, but continuously, without choline supplementation. The total level of PE methylation in cho2 cells has been estimated to be less than 20% of wild-type cells (11, 15). When cho2 mutants are grown in the absence of choline, this residual PC biosynthesis via PE methylation is, therefore, sufficient to maintain a PC content less than one-third of the wild-type level during a cell division period that is more than twice as long as that of the wild type (11, 15; Table II). The cho2, cpt1, ept1 cells have no route for PC biosynthesis other than their crippled PE methylation pathway, and they cannot increase PC production, whether choline is supplied or not. Thus, their phospholipid composition is not influenced by choline supplementation, and they grow (with or without choline) with a doubling time comparable to that of cho2 cells growing in the absence of choline (Table II). These observations concerning the growth rates of cho2 and cho2, cpt1, ept1 cells suggest that the yeast cell has some mechanism for slowing growth in response to a deficiency in PC biosynthesis. It is interesting that a comparable mechanism for sensing a deficiency in phosphatidylinositol does not exist in Saccharomyces cerevisiae. When phosphatidylinositol becomes depleted due to inositol starvation of an inositol auxotroph, the cell continues growing at a wild-type rate, producing an unbalanced metabolic condition that leads to rapid cell death (27).

Growing in the presence of choline, cho2 cells display nearly wild-type levels of PC while relying heavily on the CDP-choline pathway (Ref. 11; Table II). In cpt1, ept1 cells, no PC synthesis occurs via the CDP-choline pathway (18) but the cells achieve wild-type levels of PC (Table II), apparently by relying entirely on the PE methylation pathway. In both cases (i.e. cho2 cells growing in the presence of choline and cpt1, ept1 cells growing with or without choline), the growth rates observed are similar to growth rates achieved by wild-type cells (Table II). Thus, the high level of PC synthesis that occurs in the cho2, cki1 strain when choline is supplied appears to be invested by the cell primarily in more rapid growth as opposed to increased PE accumulation.

McMaster and Bell (18) demonstrated that cpt1, ept1 strains take up free choline and synthesize all of the intermediates of the CDP-choline pathway when grown in the presence of free choline. Both of the cpt1, ept1 mutant strains used in this study (i.e. HJ000 and HJ000 cho2) were able to take up free choline (Fig. 2) while exhibiting no incorporation of choline into PC (data not shown), as reported by McMaster and Bell (18). However, choline uptake in the cpt1, ept1, cho2 triple mutant strain (HJ000 cho2) was slightly elevated compared to the congenic double mutant strain cpt1, ept1 (HJ000) (Fig. 2). In contrast to the cpt1, ept1 strains, both of the strains carrying the cki1 mutation incorporated some choline into PC (data not shown), but the level of PC biosynthesis from free choline was quite reduced compared to the wild-type strains (25). However, incorporation of choline into PC in the cho2, cki1 double mutant strain was elevated about 5-fold compared to the cki1 single mutant strain (data not shown). The elevated level of choline uptake in cho2, cki1 and cho2, cki1, ept1, cpt1 cells, as compared to their CHO2 counterparts, suggests that the cho2 strains have enhanced activity levels in one or more steps of the CDP-choline pathway and/or choline transport. As we discussed above, the INO1 gene is inappropriately regulated in all cho2 strains. The CKi1 gene (28), the CTR1 gene, (encoding choline permease) (29), and the CPT1 gene (30) have all been shown to be subject to the same pattern of coordinated regulation that controls the INO1 gene. Thus, the enhanced uptake and incorporation of choline in cho2 strains is probably a reflection of the
The impact of the cho2 mutation on regulation of the choline transporter (29), as well as the genes encoding the coregulated enzymes of the CDP-choline pathway, including CKI1 (28), CPT1, and possibly CCT1 (encoding phosphocholinecytidyltransferase) (30).

The INO1 gene and other coregulated genes of phospholipid synthesis are regulated in response to growth phase, as well as the availability of phospholipid precursors (26). In wild-type cells, the INO1 gene is repressed during logarithmic phase if inositol alone is present. As discussed previously, choline must be present in addition to inositol for repression of INO1 to occur in cho2 cells during logarithmic phase (Figs. 3 and 5). As wild-type cells enter stationary phase, INO1 expression declines even in the absence of phospholipid precursors (26), and the same pattern was observed in the cpt1, ept1 strain. In cho2 cells grown under conditions that are derepressing for these cells in logarithmic phase (i.e. presence of inositol, absence of choline), a decline in expression of the INO1 gene was also observed as the cells entered stationary phase (Fig. 5). However, the decline in INO1 expression in cho2 cells grown in the presence of inositol and absence of choline was not as pronounced as in cpt1, ept1 cells or in wild-type cells (26) grown in the absence of inositol. Curiously, the cho2, ck1 cells, which partially repressed INO1 expression in logarithmic phase in response to inositol and choline, exhibited extreme derepression of INO1 during stationary phase under these same growth conditions. The stationary phase derepression of the INO1 gene is probably responsible for the Opi− (inositol excretion) phenotype of the cho2, ck1 strain. Indeed, the cho2, ck1 strain, which retained partial INO1 regulation in logarithmic phase, showed the most marked aberration in stationary phase regulation of INO1 of any strain examined in this study. This observation suggests that growth phase regulation of INO1 may respond to different signals than the regulation that occurs during logarithmic phase in response to inositol. Clearly, the entire mechanism of the growth phase regulation of the phospholipid biosynthetic genes merits further investigation.

At the outset of this study, we considered it possible that one of the metabolites or intermediates involved in PC biosynthesis, or the overall PC content of the cell membrane, might provide the signal for the transcriptional response to inositol during logarithmic phase. The results reported here do not support either of these models. During logarithmic phase, when growing in the presence of choline, cho2, ck1 cells exhibit PC levels that are comparable to the low proportion of PC observed in cho2, cpt1, ept1 cells (Table II). Yet in the cho2, ck1 strain, the INO1 gene was partially repressed in response to inositol during logarithmic phase when choline was supplied (Fig. 5, time point 1, compare strain 1-9C grown under the 15C and 13C growth conditions), whereas in cpt1, ept1, cho2 cells, INO1 expression was completely constitutive (Fig. 3). Thus, the proportion of PC in the cellular phospholipid composition does not appear to be tightly correlated to the cell's ability to repress INO1 in response to inositol. The cpt1, ept1 strain grown in the presence of choline was shown by McMaster and Bell (18) to take up choline and accumulate all of the CDP-choline pathway intermediates. The cpt1, ept1 and cho2, cpt1, ept1 strains used in this study were both shown to take up labeled choline (Fig. 2), consistent with the report by McMaster and Bell (18). However, in the cpt1, ept1 strain, the INO1 gene is repressed in response to inositol, whereas INO1 is completely constitutive in the cho2, cpt1, ept1 strain. Thus, the ability to take up choline and produce the intermediates of the CDP-choline pathway does not appear to be correlated to the ability to repress the INO1 gene in response to inositol. Similarly, production of wild-type levels of the intermediates in the PE methylation pathway does not appear to correlate to the regulation. INO1 repression in cho2 mutants is restored by choline supplementation, a condition that stimulates PC production via the CDP-choline pathway but does not restore PE methylation. Indeed, regulation of INO1 in response to inositol appears to be correlated only to overall PC biosynthesis and not to the route of synthesis or to the level of production of any intermediate in either of the two routes of synthesis. Rather, the results reported here suggest that yeast cells are able to monitor some process linked to the actual synthesis of PC or its effect on cell growth, independent of the route by which PC is produced. When PC synthesis is growth limiting, the combination of choline and inositol results in INO1 repression. If PC content is not growth limiting, as in wild-type cells or cpt1, ept1 cells, then repression occurs in response to inositol alone. The mechanism by which PC biosynthesis is monitored and by which it results in transmission of a signal capable of controlling synthesis of the structural genes of phospholipid biosynthesis remains to be elucidated.

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