Regulation of Phospholipid Biosynthetic Enzymes by the Level of CDP-Diacylglycerol Synthase Activity*

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The major phospholipids in yeast, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine (PS), and phosphatidylinositol (PI), are synthesized by two branches of the phospholipid biosynthetic pathway that diverge from the common liponucleotide precursor CDP-diacylglycerol (CDP-DAG) (1–3). Synthesis of amine-containing lipids starts with the conversion of CDP-DAG and serine to phosphatidylserine (PS), whereas phosphatidylinositol (PI) is formed from CDP-DAG and inositol (derived from inositol 1-phosphate). In this study the regulation of PS synthase from CDP-DAG and inositol (derived from inositol 1-phosphate) was investigated.

Studies of the regulation of phospholipid biosynthesis by inositol demonstrate that the INO1 and CHO1/PSS genes are derepressed in wild type cells grown in medium lacking inositol and choline (or ethanolamine), and repressed when these precursors of phospholipid biosynthesis are present (9, 10); choline and ethanolamine are precursors in the DAG-dependent pathway leading to phosphatidylcholine and phosphatidylethanolamine biosynthesis (1, 3). The regulation of INO1 and CHO1/PSS gene expression by inositol and choline has been shown to occur at the level of mRNA abundance (11, 12) and is mediated by the 5′ cis-acting UAS_{INO} (upstream activation sequence) and the trans-acting INO2-INO4-OP1 regulatory gene products (10, 12). The INO2 and INO4 genes encode positive regulators of transcription that are required for derepression in response to inositol and choline deprivation. Ino2p is the transcriptional activator that is dependent on dimerization with Ino4p for its binding to the UAS_{INO} sequence. Strains carrying ino2 or ino4 mutant alleles fail to express the INO1 gene product and thus need inositol supplement for growth (13). INO2 expression is also coordinately regulated by inositol (14). The OP1 gene product is required for repression of gene expression in response to inositol and choline supplementation. The op1 mutant strains constitutively overexpress the INO1 and CHO1 genes (15). Studies using the CAT reporter gene fused to the endogenous promoter of the OP1 gene or the INO2 gene suggest that OP1 is required for the initial response to inositol but that regulation of INO2 expression establishes the magnitude and cooperativity of the response (14, 16).

Expression of the PIS1 gene, as with the INO4 or OP1 genes, does not respond to inositol regulation, although a UAS_{INO} sequence is also found 5′ to the PIS1 open reading frame (17). Instead, PIS1 expression is regulated at the transcriptional level by carbon sources in growth medium (18). Relative to cells grown in a glucose medium, expression of the PIS1 gene is reduced when cells were grown in a medium containing glycerol and elevated when cells were grown in a medium containing galactose. Carbon source regulation involves the binding of Mcm1 protein to the cis-MCEs sequences and the involvement of the two-component regulatory gene SLN1 (18).

We previously reported the isolation of the CDS1 gene encoding CDP-DAG synthase activity in yeast (19). By gene inactivation, we showed that this gene is essential for spore germination and vegetative cell growth. Overexpression of CDP-DAG synthase activity results in an elevation in the apparent initial rate of synthesis of PI relative to PS. A reduction of CDP-DAG synthase activity to 10% of the wild type level has an opposite effect on the above phospholipid biosynthetic steps. Reduced synthase levels also resulted in inositol excretion. By characterizing the cdg1 mutant (20), we found a point mutation within the CDS1 gene that results in the cdg1 phenotype of pleiotropic deficiencies in phospholipid biosynthesis, including a reduced level of CDP-DAG synthase activity, an elevated

* The abbreviations used are: PS, phosphatidylserine; PI, phosphatidylinositol; DAG, diacylglycerol; CSM, complete synthetic medium.

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phosphatidic acid content, a constitutive elevated level of inositol 1-phosphate synthase activity, and a reduced cellular CDP-DAG synthase level. All of these results are suggestive of alteration of expression of the INO1 and CHO1/PSS genes at reduced cellular CDP-DAG synthase levels.

Cloning of the CDS1 gene affords the opportunity to study the role of CDP-DAG synthase and its product in the regulation of phospholipid metabolism. In this study, we examined the regulation of phospholipid biosynthetic enzymes by CDP-DAG synthase activity. We found that the increased PS synthase activity and the inositol excretion in response to reduced CDP-DAG synthase activity was due to an increased steady-state level of CHO1/PSS and INO1 mRNA transcripts, respectively, whereas the parallel decrease in PSI synthase activity was not a result of a change in the PSI1 mRNA transcript level. However, regulation of expression of the CHO1/PSS and INO1 genes in response to CDP-DAG synthase activity levels did not correlate with factors that regulate the expression of these genes in response to inositol and choline.

EXPERIMENTAL PROCEDURES

Materials—Simple salts, buffers, and amino acids were purchased from Sigma and Fisher. YEP broth and synthetic media for yeast growth and selection were from Bio 101, Inc. Yeast nitrogen base without amino acids was from Difco Laboratories. Trizol reagent was a product of Life Technologies, Inc. Positively charged nylon membranes, Genius 1 kit, and reagents for nonradioactive detection of nucleic acid were purchased from Boehringer Mannheim. Radiochemicals were obtained from Amersham Corp. Oligonucleotides were prepared commercially by Genosys Biotechnologies. The Gene Amp polymerase chain reaction reagent kit was from Perkin-Elmer Cetus. Qiaex II gel extraction kit was from Qiagen Inc. CDP-DAG was synthesized by reaction of MgCl2, 1% Triton X-100, 1.0 mM [5-3H]CTP (9,000 cpm/nmol), 0.5 mM [3-3H]serine (10,000 cpm/nmol), 0.5 mM [3-3H]inositol (10,000 cpm/nmol) into PI in 50 mM Tris–HCl buffer, pH 8.0, 0.2 mM MnCl2, 0.2 mM CDP-DAG, 0.25% Triton X-100, 0.5 mM t-[-3H]serine (10,000 cpm/nmol), and 75 μg of membrane protein in a total volume of 0.1 ml (29). PS synthase activity was measured by the incorporation of radiolabel into PS in 50 mM Tris–HCl buffer, pH 8.0, 0.6 mM MnCl2, 0.2 mM CDP-DAG, 0.25% Triton X-100, 0.5 mM t-[3H]serine (10,000 cpm/nmol), and 75 μg of membrane protein in a total volume of 0.1 ml (30). All assays were carried out at 30 °C for either 5 min (CDP-DAG synthase) or 20 min (PS and PI synthases).

Probes for RNA analyses—Double-stranded DNA probes were used for Northern blot analysis. The probe for CDS1 detection was a BstEII-BglII fragment from plasmid pSDG1 (19). The remaining probes used in this study were polymerase chain reaction products generated using YPH102 genomic DNA as a template and the polymerase chain reaction primers listed in Table I designed according to the DNA sequences of CHO1/PSS (5), INO1 (4), INO2 (31), PIS1 (32), PIS2 (7), and TCM1 (33). Each probe was subcloned into plasmid pBluescript KS and verified to be correct by DNA sequencing before being labeled by random priming and incorporation of digoxigenin-dUTP using the Genius 1 kit according to the manufacturer's instructions. Concentrations of labeled probes for individual detections were 5 ng/ml TCM1, 20 ng/ml CHO1, INO1, PIS1, and 50 ng/ml NO2, PIS2.

RNA Analyses—Yeast cells were harvested at different time points and were frozen at −80 °C before RNA preparation. Total RNA was isolated from these samples by a glass-bead disruption/Trizol extraction procedure. Yeast pellets from 10 ml of mid-log phase cell culture were mixed with 0.2 ml of prechilled silicon beads (diameter, 0.3 mm) and 1 ml of Trizol and disrupted in a Mini-Beadbeater (Bioective Products) by a 2-min burst at 2800 rpm. The aqueous phase was then separated, and RNA was precipitated according to the instructions for RNA isolation (Life Technologies, Inc.). Typically, 30 μg of RNA was run on a 1.5 agarose gel overnight, transferred to a nylon membrane, and UV cross-linked to the nylon membrane using a Stratalinker device (Stratagene). Denatured probe was hybridized to the membrane-bound RNA, detected with anti-digoxigenin antibody, visualized by autoradiography according to the Genius System User's Guide (Boehringer Mannheim), and quantitated by densitometry.

RESULTS

Cell Growth of cds1 Mutant at Low CDP-DAG Synthase Activity—As previously reported (19), the cds1::TRP1 strain (cds1 mutant) depends on the expression of a plasmid-borne copy of CDS1 for viability. Introduction of a single copy plasmid, pSDG2 (tGAL1-CDS1), into a cds1 mutant supported cell growth in galactose-containing induction medium, but not in a glucose-containing noninduction medium where CDP-DAG synthase activity was undetectable in cell extracts at the point of cell arrest. To study the cellular response at low CDP-DAG synthase levels, we further characterized the growth phenotype of this mutant. Mutant cds1 cells grew normally in galactose medium compared with the wild type strain YPH102 because the growth of the former strain was fully supported by overexpression of the plasmid-borne CDS1 gene. Cell growth rate gradually decreased as indicated by the increase in the doubling time over the first 16 h after the cells were shifted to a glucose medium followed by arrest of growth by 24 h (Fig. 1A). There was a significant difference in cell viability between 16 and 24 h of growth. About 50% of the cells were viable after 16 h versus less than 20% after 24 h as evidenced by colony formation on galactose-containing plates (Fig. 1B), indicating that viability of cells grown at 30 °C was irreversible after prolonged periods below a threshold level of CDP-DAG synthase activity.

PS and PI synthase activities and INO1 expression are regulated by CDP-DAG synthase activity. CDP-DAG synthase activity in strain YSD900/pSDG2 grown in galactose induction medium was 10-fold as high as the wild type level (strain YPH102). This activity was reduced after cells were
shifted to the glucose noninduction medium, reaching the wild type level within 8 h (approximately 3 generations), and only about 10–15% of that level after 16 h of cell growth (Fig. 2A). PI synthase activity also followed the CDP-DAG synthase activity trend starting at 40% above wild type level and dropping first to 100% and then to 30% of the wild type level (Fig. 2C). PI synthase activity in wild type yeast YPH102 was higher when cells were grown in galactose medium than in glucose medium, but there was only a 1.4-fold change in the wild type strain as compared with about a 4–5-fold change in the mutant strain. PS synthase activity, however, showed the opposite trend, beginning at 40% of the wild type level, increasing first to the wild type level and then to almost 3-fold as high as the wild type level in the cds1 mutant (Fig. 2B). Carbon source did not affect PS synthase or CDP-DAG synthase activities in the wild type strain.

We next asked whether these biochemical changes were a result of regulation of existing enzyme activity or an alteration...
in steady-state mRNA levels. Expression of the CHO1/PSS, INO1, and PIS1 genes was quantitated by Northern blot analysis of total cellular RNA from both the cds1 mutant (carrying pSDG2) and the wild type strain YPH102. The results were normalized using a TCM1 probe specific for ribosomal protein L3 (33) previously shown to be unaffected by factors affecting the expression of phospholipid biosynthetic enzymes (12).

Transcripts of both the target genes and the TCM1 gene in the cds1 mutant were significantly lower at the 16-h time point than at the earlier time points, which indicated that the cells were very sick by this time; therefore, we chose time points from 0 to 12 h, where there was no change in the TCM1 mRNA levels. As shown in Fig. 3, the amount of CHO1/PSS transcript increased gradually from below the wild type level in the induction medium to almost 3 times as high as the wild type level, paralleling the reduction of cellular CDP-DAG synthase activity. This increase was in agreement with the change of PS synthase activity over the course of the experiment. The INO1 mRNA level was higher in cells grown in galactose medium than in glucose medium in both strains, YPH102 and YSD90A (0 h versus 4 h). However, there was no increase in the mutant strain relative to the wild type strain until CDP-DAG synthase activity was well below the wild type level (Fig. 3), correlating with our previous observation that the cds1 mutant excreted inositol into the growth medium only when cellular CDP-DAG synthase activity was low (19, 21). The PIS1 mRNA level remained the same (Fig. 3) despite the significant reduction in PS synthase activity over the course of the experiment. We were unable to measure CDS1 transcript levels in the cds1 mutant extracts due to interference by pSDG2 plasmid DNA.

Effect of Human CDP-DAG Synthase Activity on CHO1/PSS and INO1 Expression—Because both CDP-DAG synthase protein and activity were overexpressed in the induction medium, the above regulation could be due to either the amount of activity, which determines the cellular CDP-DAG level, or the amount of CDS1 gene product. To address this point, we tested the effect of a human CDP-DAG synthase, isolated from a leukocyte cDNA library, on the transcription of the above genes. GAL1 promoter-controlled overexpression of this gene complements the growth phenotype of the yeast cds1 mutant (26). However, CDP-DAG synthase activity is only 20–30% of the wild type yeast level, resulting in inositol excretion even in the induction medium. This transformant stopped growing in noninduction medium after 1–2 generations of growth (data not shown), consistent with synthase activity being near the threshold level for growth. Cells were grown in galactose medium to mid-log phase, shifted to a glucose medium, and grown for an additional 3 h. Total RNA was isolated from these cells and was used for quantitation of CHO1/PSS and INO1 transcripts by Northern blot analysis and normalized to the TCM1 transcript. Both transcripts were higher in the cds1 mutant than in the wild type control (Fig. 4). A shift from galactose to glucose medium did not significantly affect levels of these transcripts, suggesting that both CHO1/PSS and INO1 were fully overexpressed at 20–30% of the wild type CDP-DAG synthase level. This result argues that it is CDP-DAG synthase activity, and not synthase protein level, that regulates CHO1/PSS and INO1 expression.

Transcription of INO2 and OPI1 as a Function of CDP-DAG Synthase Activity—CDP-DAG synthase regulation of CHO1/PSS and INO1 expression showed surprising similarity to the response of these two genes to inositol and choline, which is mediated by the INO2-INO4-OP11 gene products (9, 10). To determine the potential involvement of these regulatory factors in the alteration in gene expression brought about by changes in CDP-DAG synthase activity, we examined the levels of INO2 and OPI1 transcripts in the cds1 mutant by quantitative Northern blot analysis. The data in Fig. 5 show that transcripts of INO2 and OPI1 were the same in both wild type yeast and the cds1 mutant regardless of the change of CDP-DAG synthase activity in the latter strain. Therefore, regulation of gene expression by CDP-DAG synthase activity is not correlated with changes in expression of regulatory genes that respond to inositol.

Regulation of CDS1 Expression by Inositol—Expression of CDP-DAG synthase activity, like that of the other enzymes in the phosphatidylcholine biosynthetic pathway, is coordinately regulated by inositol and choline in the growth medium (34). However, the molecular mechanism for the regulation of this enzymatic activity has not been established. Because a cis-acting UASINO sequence has also been found 5' to the CDS1 gene (19), we examined whether CDS1 expression is also regulated by inositol.

Wild type strain YPH102 and the ino1 mutant MC13 were
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**FIG. 5.** INO2 and OP1 mRNA transcript levels as a function of CDS1 gene expression. Wild type strain YPH102/YCpGAL and the mutant cds1 strain YSD900ApSDG2 were grown and analyzed for mRNA levels as described in Fig. 3. Values reported are the average of two independent experiments.

First grown in a synthetic medium containing 60 μM inositol and 1 mM choline (+ino). Cells were shifted to inositol- and choline-free media (−ino), and growth was continued for 4 h before harvesting. Transcripts of CDS1 and INO2 were assayed by quantitative Northern blot analysis and normalized to the TCM1 transcript. In both wild type yeast and ino1 mutant strains, INO2 transcript levels were low in cells growing in +ino medium as compared with −ino medium, and the levels of the CDS1 transcript followed those of INO2 (Fig. 5). These results indicate that regulation of CDS1 expression by inositol and choline is correlated with the transcript level of the cognate activator gene INO2, which is also regulated by precursors of phospholipid biosynthesis.

**DISCUSSION**

The results reported here demonstrate that phospholipid biosynthetic activity can be regulated by variations in CDP-DAG synthase activity. A shift of the cds1 mutant from induction to noninduction medium abolished the expression of the plasmid-borne CDS1 gene and caused changes over a 100-fold range in CDP-DAG synthase specific activity from 10-fold above to about 10% that of the wild type level. Although CDP-DAG synthase specific activity was reduced, total activity in the culture remained the same, which argues that the decreasing specific activity was due to dilution, not inactivation, of this enzyme. Such a change in enzymatic activity would dramatically affect the potential synthetic capability for CDP-DAG, a substrate for both PS and PI biosynthesis. In our previous studies (19), we found that down-regulation of plasmid-borne CDS1 expression in a cds1 mutant resulted in a reduction in both the initial rate of biosynthesis and the steady-state level of PI and an opposite increase with respect to PS; we also observed significant excretion of inositol. In this study we showed that the molecular basis for such alterations in phospholipid composition is a result of changes in the effective enzymatic activities of the two branch enzymes, PS synthase and PI synthase, and an apparent increase in inositol 1-phosphate synthase.

The most novel finding in this study was that PS synthase and inositol 1-phosphate synthase activities are regulated at the transcriptional level, whereas PI synthase is regulated posttranscriptionally in response to changes in CDP-DAG synthetic capability. This is the first indication that these important phospholipid biosynthetic enzymes are regulated in response to an upstream enzymatic activity in the phospholipid biosynthetic cascade. Although regulation of expression of both

**FIG. 6.** Expression of the CDS1 and INO2 mRNA transcripts as a function of inositol and choline in the growth medium. Wild type strain YPH102 and the inositol auxotroph MC13 grown to mid-log phase in synthetic medium with 2% glucose and supplemented with 60 mM inositol and 1 mM choline (+ino) were washed with synthetic medium without inositol and choline supplementation. Growth was continued in the same inositol- and choline-free medium (−ino) for 4 h. Total RNA preparation and Northern blot analysis were performed as described under “Experimental Procedures.” The amount of each transcript was normalized to that of the TCM1 gene. The reported target gene/TCM1 transcript ratio was normalized to the target gene/TCM1 transcript ratio (set to 1.0) of the wild type strain grown in inositol-free medium; n.d. indicates not detectable. Values reported are the average of two independent experiments.

CHO1/PSS and INO1 by CDP-DAG synthase activity is qualitatively similar, there are significant differences in the response of these two genes. The increase in the steady-state level of the CHO1/PSS gene transcript followed the dilution of CDP-DAG synthase activity during cell growth and division (Fig. 3). On the other hand, INO1 expression was not further repressed over wild type levels by overproduction of CDP-DAG synthase activity and was only derepressed when synthase levels were reduced below wild type levels.

Previous results suggested that the PI synthase level is not subject to coordinate regulation by inositol and choline (17) but is primarily regulated by the available concentration of its substrates, inositol and CDP-DAG (8). The results reported here demonstrate that PI synthase activity also responds to the cellular CDP-DAG synthase activity. Overall, PI synthase activity was higher in cells grown in galactose medium than in glucose medium, consistent with the recent report (18) on the effects of carbon source on the PIS1 transcript levels. However, Northern blot analysis did not explain the decrease in PI synthase activity that followed the trend in CDP-DAG synthase activity during cell growth and division (Fig. 3). On the other hand, INO1 expression was not further repressed over wild type levels by overproduction of CDP-DAG synthase activity and was only derepressed when synthase levels were reduced below wild type levels.
agreement with studies from other groups demonstrating that inositol regulates gene expression via the INO2-INO4-OP1 regulatory genes (1–3). Inositol regulation of CDS1 expression parallels the amount of INO2 transcript, as does the expression of CHO1/PSS and INO1 (14). Our results are the first report of coordinate and unique regulation by CDP-DAG synthesize activity levels of several genes related to phospholipid metabolism. Clearly, INO2-INO4-OP1 regulatory genes are not the only ones that control expression of phospholipid biosynthetic genes. INO1 transcription is also sensitive to mutations that affect the more general transcriptional apparatus, such as SWI1, SWI2, and SWI3, and these mutant strains are inositol auxotrophs (37). Mutation of the SIN3 gene (also known as CPE1/UME4/RPD1/GAM2/SD11) also affects a variety of genes and causes constitutive derepression of INO1 transcription (38–41). Expression of INO1 is also regulated by SIN1 gene product (37). It is not clear what senses a change in CDP-DAG synthesize activity and how this signal is transmitted downstream to regulate the phospholipid biosynthetic pathway. However, CDP-DAG itself has been shown to inhibit a 45-kDa PI 4-kinase activity in a dose-dependent manner (42). The latter catalyzes the committed step in the synthesis of the phosphoinositides PIP and PIP2 in Saccharomyces cerevisiae (43). PIP2 or its hydrolysis products play an essential role in yeast proliferation (44). It is thus possible that CDP-DAG levels could play an important role in the regulation of phospholipid metabolism and cell growth via effects on the phosphoinositide pools (42). The observation that CDP-DAG synthesize activity perturbs phospholipid metabolism by affecting the expression and the level of phospholipid biosynthetic enzymes is nevertheless significant. These results demonstrate the complexity in the control of phospholipid metabolism necessary to ensure the efficient response of the cell to changes in the environment.

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