Regulation of Phosphatidylglycerophosphate Synthase by Inositol in \textit{Saccharomyces cerevisiae} Is Not at the Level of \textit{PGS1} mRNA Abundance*

Quan Zhong and Miriam L. Greenberg‡

From the Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202

Phosphatidylglycerophosphate synthase catalyzes the committed step in the synthesis of the mitochondrial phospholipid cardiolipin. We showed previously that phosphatidylglycerophosphate synthase activity in \textit{Saccharomyces cerevisiae} is increased in conditions favoring mitochondrial development and during growth in the absence of inositol. Interestingly, the regulatory effects of inositol were not altered in \textit{ino2}, \textit{ino4}, or \textit{opi1} mutants suggesting that regulation in response to inositol is not at the level of gene transcription. We report here that steady state mRNA levels of the \textit{PGS1} gene, which encodes phosphatidylglycerophosphate synthase, were not altered by inositol or choline. Growth in the presence of the inositol-depleting drug valproate led to an increase in phosphatidylglycerophosphate synthase activity unaccompanied by increased \textit{PGS1} mRNA. \textit{PGS1} mRNA abundance was not decreased in \textit{ino2} or \textit{ino4} mutants and was unaffected in an \textit{opi1} mutant. Therefore, regulation of phosphatidylglycerophosphate synthase by inositol is not mediated at the level of mRNA abundance and does not require the \textit{INO2-INO4-OP11} regulatory circuit. \textit{PGS1} was increased in glycerol/ethanol compared with glucose media and was maximally expressed as cells entered the stationary phase. Deletion of the mitochondrial genome did not affect \textit{PGS1} expression. Thus, whereas inositol controls phosphatidylglycerophosphate synthase activity, regulation of \textit{PGS1} expression occurs primarily in response to mitochondrial development cues.

Cardiolipin (CL), a unique phospholipid with dimeric structure, is ubiquitous in eukaryotes and predominantly found in the mitochondrial inner membrane (1). It plays a role in mitochondrial bioenergetics by optimizing activities of enzymes in the oxidative phosphorylation pathway, including complexes I, III, IV, and V (2–5), and the ADP-ATP carrier (6–8). It is also involved in mitochondrial biogenesis possibly via assisting protein import into mitochondria (8) and maintaining optimal mitochondrial internal structure (9). As a reflection of the importance of CL, its synthesis is highly regulated in response to various factors (1). Fully understanding the regulation of CL biosynthesis will provide important insight into the function of CL in cellular processes.

Phospholipid biosynthetic pathways have been extensively characterized in \textit{Saccharomyces cerevisiae}. Phospholipid synthesis proceeds via a three-branched pathway from the central intermediate CDP-DAG. A general regulation pattern has been observed for most structural genes encoding the enzymes in the PC and inositol biosynthetic pathways including \textit{INO1, CHO1}, \textit{PSD1}, \textit{CHO2}, and \textit{OP13} (10, 11). The central feature of this regulation is repression by inositol and choline. A consensus sequence inositol choline-responsive element, also termed UAS\textsubscript{INO}, was found in the promoter regions of genes coordinately regulated by inositol. In the absence of inositol, heterodimers of the positive transcriptional regulators Ino2p and Ino4p bind to the UAS\textsubscript{INO} to activate transcription. In the presence of inositol, the \textit{OP11} gene product acts to repress transcription.

Transcriptional control of UAS\textsubscript{INO}-containing genes is uniquely affected by the growth phase. Expression is maximal during logarithmic growth and is reduced as cells enter the stationary phase (11). Repression of these genes in the stationary phase may be correlated with the repression mechanism involving the two major phospholipid precursors, inositol and choline, because both mechanisms of regulation share two common characteristics (12). First, they require a functional UAS\textsubscript{INO} sequence in the promoter region of targeted genes, which may serve as the cis-element for the activation of transcription. Second, ongoing PC synthesis is essential for the repression of gene expression.

The biosynthesis of CL is conserved in all eukaryotic organisms. It occurs via three enzymatic reactions (1). PGPS catalyzes the committed step of CL synthesis, formation of PGP from CDP-DAG and glycerol-3-P. PGP phosphatase dephosphorylates PGP to PG. CL synthase catalyzes the final step of CL synthesis, condensation of CDP-DAG and PG to form CL. In contrast to the phosphatidylinositol and PC branches, biosynthesis of CL occurs solely in the mitochondria. Therefore, regulation of CL synthesis may be expected to respond not only to cross-pathway control by inositol and choline but also to cues affecting mitochondrial development.

Like many enzymes in the phosphatidylinositol and PC branches, PGPS, the committed enzyme of CL synthesis, is subject to coordinate control by the phospholipid precursors inositol and choline (13). However, the mechanism of regulation at the level of gene expression has not been elucidated. Previous studies suggested that regulation of the CL pathway by inositol occurs via a mechanism that differs from that of the PC and inositol biosynthetic pathways. Activity of the first pathway enzyme, PGPS, is reduced ~70% when cells are grown
in the presence of exogenous inositol in *S. cerevisiae*. However, enzyme activity is not affected in *ino2, ino4*, or *opi1* mutants, suggesting that control by inositol is not mediated at the level of PGS1 transcription (13). Furthermore, in contrast to enzymes encoded by UASINO-containing genes, PGS activity increases 2–4-fold in the early stationary phase (14). These observations are consistent with the lack of a functional UASINO sequence in the PGS1 promoter. Nevertheless, in an analysis of PGS1 expression using a plasmid-borne lacZ reporter construct under control of the putative PGS1 promoter, β-galactosidase activity was decreased 2–fold during growth in the presence of inositol. This regulation was attributed to a potential UASINO element 284 bp 5′ to the PGS1 5′ open reading frame (15). However, this element has an UASINO that has an A in place of a T in the critical third position, which was shown to be functionally inactive (16). It is likely that the reporter construct on a multicopy plasmid does not accurately reflect PGS1 regulation as it occurs in the genome.

Regulation of the CL biosynthetic pathway by inositol has been observed in higher eukaryotic organisms as well as yeast. Addition of inositol to lung microsomes inhibited PG synthesis up to 94% (17), and PG levels increased more than 10-fold during inositol starvation in two independently isolated inositol-auxotroph Chinese hamster ovary cell lines (18, 19). However, the underlying mechanisms that mediate such regulation remain elusive.

Identification of the *S. cerevisiae* structural genes PGS1 (20, 21) and CRD1 (22–24) coding for enzymes in the CL biosynthetic pathway enables the molecular analyses of the regulation of CL biosynthesis. To determine how PGS1 expression is regulated we measured PGS1 mRNA abundance directly. Our studies revealed that regulation of PGS1 activity by inositol is not mediated at the level of mRNA abundance, and it does not require the INO2-INO4-OP1 regulatory circuit. Rather, transcriptional regulation of PGS1 occurs primarily by derepression during the stationary growth phase when mitochondrial development is increased.

**EXPERIMENTAL PROCEDURES**

**Materials**

All chemicals used were reagent grade or better. [α-32P]UTP was purchased from PerkinElmer Life Sciences. CDP-DAG was obtained from Life Science Resources, and 1-[[2-3H]glycerol 3-phosphate (20 Ci/mmol) was purchased from American Radiolabeled Chemicals. The PCR was performed using the MasterTaq kit from Eppendorf. RT-PCR was performed using the Access RT-PCR system from Promega. The Wizard Plus Miniprep DNA purification system, the pGEM-T Easy Vector System, and the Riboprobe System kit were from Promega. All other buffers and enzymes were purchased from Sigma. Glucose, yeast extract, and peptone were purchased from Difco.

**Methods**

**Yeast Strains and Growth Media**—The yeast *S. cerevisiae* strains used in this work are listed in Table I. Yeast strains were grown at 30°C. Synthetic minimal medium contained necessary amino acids (histidine (20 mg/liter), leucine (60 mg/liter), tryptophan (20 mg/liter), and uracil (20 mg/liter)), vitamins, salts (essentially components of Difco Vitamin-free yeast base without amino acids), and either glucose (2%) or glycerol (3%) plus ethanol (0.95%) as carbon source. Complex YPD medium contained yeast extract (1%), peptone (2%), and glucose (2%). Solid medium contained agar (2%) in addition to the above.

**Plasmid Construction**—A 1143-bp PGS1 coding sequence was amplified from yeast chromosomal DNA using PGS1 5′ (5′-AGGACTCGTT-TGCTCCAACT-3′) and PGS1 3′ (5′-TACGCTTTCCTGCACTCTC-3′). The PCR reaction products were cloned into the pGEM-T Easy Vector. The resulting recombinant plasmid, pGEM-PGS1, was linearized with ClaI for the synthesis of the PGS1 riboprobe.

**Disruption of PGS1**—The *S. cerevisiae* PGS1 gene was replaced by TRP1 using a one-step gene replacement strategy in which 1220 bp from the center of the open reading frame beginning 263 bp after the start codon was replaced with 862 bp of the TRP1 gene. Transformants of diploid cells were selected on Trp− drop out synthetic medium. Dissection of tetrad produced haploid strains that contain only one copy of the disrupted PGS1 gene. Disruption of PGS1 was confirmed by PCR using primers against the TRP1 sequence (Trp5′, 5′-CCAAACTTCCAACAATGGA-3′) and the sequence 3′ to the PGS1 coding sequence (PGS2193, 5′-AGGACATTCCATTACCTCA-3′). Isolation of rho0 Mutants—Isolation of ethidium bromide-induced rho0 mutants was performed as described previously (25). Briefly, cells were grown to saturation in synthetic minimal medium with 2% glucose and 25 µg/ml ethidium bromide (filter-sterilized). A second culture was inoculated from the first in the same medium and grown to saturation. Single colonies were streaked on YPD. Essentially all single colonies were rho0 mutants. The resulting strains were crossed with a series of rho− tester strains 104, 105, 106, and 107. Failure to complement any of the tester strains confirmed the loss of mitochondrial genome.

**Quantitative Reverse Transcriptase-PCR (RT-PCR)**—Yeast strains were grown to the early stationary growth phase in synthetic medium...
with 2% sucrose. Total RNA was isolated by hot phenol extraction (26) and treated with DNase to avoid DNA contamination. RT-PCR was performed using the RT-PCR Access System from Promega. PGS1 mRNA was amplified using primers RTpgs15 (5′-AAGCGGAGCT-GAGTTGGTG-3′) and RTpgs13 (5′-ATGACACCCCTGATGTTG-3′). The internal control ACT1 was amplified using primers RTact15 (5′-TAAACGTTGCGTGTTGGA-3′) and RTact13 (5′-TTGGGGT-GAAACGATAGTGG-3′). Standard reactions used 30 cycles, 50 pmol of each primer, and 100 ng/µl RNA in 50 µl. RT-PCR products were separated by agarose electrophoresis. The intensity of DNA products was analyzed using Adobe Photoshop software. The products of PGS1 from each RNA preparation were normalized to ACT1.

Northern Analysis—Cells were grown in minimal synthetic medium with either glucose or glycerol/ethanol as a carbon source. Inositol, choline, or valproate was added when indicated. Cells were harvested at the indicated times; RNA was isolated by hot phenol extraction (26) and fractionated on an agarose gel and then transferred to a nylon membrane. The blots were hybridized with 32P-labeled riboprobes. 32P was quantified by Phosphorimaging analysis using ImageQuant software. Expression of PGS1 (○) and INO1 (●) was normalized by comparison with TCM1. Data represent the average of two independent experiments.

RESULTS

Identification of PGS1 Transcripts—The PGS1 transcript was identified by comparison of RNA from wild-type and pgs1Δ strains. Total RNA from pgs1Δ and isogenic wild-type strains was isolated and hybridized with a PGS1 riboprobe. A 1.7-kb PGS1 mRNA was observed only in RNA from the wild-type cells (Fig. 1A). RT-PCR analysis of total RNA isolated from the
wild-type and pgs1Δ mutant confirmed this result. As seen in
Fig. 1B, PGS1 was amplified only from wild-type RNA.

PGS1 Expression Peaks in the Early Stationary Phase—
Maximum activities of CDP-DAG synthase, PS synthase, and
the phospholipid N-methyltransferases were found in the ex-
ponential growth phase in cells grown in complete synthetic
medium (29). Transcription of the structural genes coding for
those enzymes showed a similar expression pattern. In con-
trast, PGPS enzyme activity increased as cells enter the sta-
tionary phase (14). Consistent with enzyme activity maximal
PGS1 mRNA levels were observed as cells entered the station-
ary phase (Fig. 2B) in contrast to the UASINO-containing gene
INO1, which was fully derepressed in the logarithmic phase
and decreased as cells entered the stationary phase (Fig. 2B).

PGS1 mRNA Abundance Is Not Altered by Inositol and Cho-
line—As discussed above, PGPS enzyme activity is decreased
in the presence of inositol. To determine whether the effect of
inositol is because of transcriptional regulation, the effect of
inositol on the steady state level of PGS1 mRNA was examined
in cells grown to the early stationary growth phase. PGS1
mRNA abundance was not altered in cells growing in the pres-
ence of exogenous inositol or choline (Fig. 3A). In contrast,
expression of INO1, which is known to be repressed by inositol,
was reduced more than 10-fold (Fig. 3B). INO1 expression was
measured in the logarithmic phase, which is the time of max-
imal expression of this gene (see Fig. 2). We showed previously
that PGPS activity was decreased within minutes after inositol
supplementation (13). We looked at PGS1 expression as a func-
tion of time after addition of inositol. PGS1 mRNA levels did
not decrease after the addition of inositol, as seen in Fig. 4A,
although PGPS enzyme activity was significantly decreased
within 60 min following supplementation with inositol (Fig.
4C). In contrast, expression of INO1 was fully repressed within
30 min following the addition of inositol (Fig. 4B).

Effect of Valproate-induced Inositol Depletion on PGS1 Ex-
pression—VPA is an 8-carbon branched fatty acid used in the
treatment of bipolar disorder. VPA causes a decrease in intra-
cellular inositol mass leading to a dramatic increase in expres-
sion of INO1 in S. cerevisiae (30). To examine the effect of
inositol depletion on PGS1 expression PGS1 mRNA abundance
was measured in cells grown in the presence of VPA. As shown
in Fig. 5A, VPA did not lead to an increase in PGS1 mRNA
levels under conditions that led to more than 10-fold increase
in INO1 mRNA (Fig. 5B). Interestingly, the rate of synthesis of
PG was increased up to 3-fold in the presence of VPA2 Consis-
tent with this observation, cells grown in the presence of
VPA exhibited a 2-fold increase in PGPS activity (Fig. 5C).
Therefore, inositol depletion by VPA led to an increase in PGPS

\[ \text{PGS1 Expression Peaks in the Early Stationary Phase—} \]

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enzyme activity with no concomitant increase in PGS1 expression.

**Derepression of PGS1 Expression in the Positive Regulatory Mutants ino2 and ino4** —The INO2 and INO4 gene products are required for the derepression of UAS_INO2-containing phospholipid biosynthesis genes in the absence of exogenous inositol. A mutation in either INO2 or INO4 significantly reduces the expression of UAS_INO2-containing genes (10). The ino2 and ino4 mutants require exogenous inositol for growth because these mutants fail to express the INO1 gene product required for de novo inositol synthesis (31). In contrast, wild-type levels of PGPS activity were found in ino2 and ino4 mutants suggesting that INO2 and INO4 are not required for derepression of PGPS (13). To address this possibility, PGS1 expression was characterized in ino2 and ino4 mutants. PGS1 expression was observed at derepressed levels in both mutants in all conditions (Fig. 6A) in contrast to the INO1 gene, which was barely detectable in these mutants (Fig. 6B). Interestingly, PGS1 expression was increased 2–3-fold in the ino4 mutant, which suggests a possible negative regulatory role of the INO4 gene product in transcription of PGS1.

**The Steady State Level of PGS1 mRNA Is Not Altered in the opi1 Mutant** —Disruption of the negative regulatory gene OPI1 results in constitutive overexpression of UAS_OPI1-containing genes (10). The opi1 mutant excretes inositol (32) because of overexpression of the INO1 gene even in inositol-free medium. PGPS activity, however, was reduced in the opi1 mutant (13) suggesting that OPI1 does not cause repression of PGS1 expression. PGS1 mRNA abundance was measured in the opi1 mutant. As seen in Fig. 7A, wild-type levels of PGS1 were observed in the opi1 mutant in the absence of inositol. In contrast, INO1 levels were more than 20-fold higher in the opi1 mutant than in the wild type in inositol-free medium (Fig. 7B). These findings and those in Fig. 6 demonstrate that transcriptional control of PGS1 is not mediated by the INO2-IN4-OPI1 regulatory circuit.

**Effect of Mitochondrial Development on PGS1 mRNA Abundance** —PGPS activity is regulated by factors affecting mitochondrial development, such as growth phase, carbon source, and mitochondrial genome (14). Only 30–70% of wild-type levels of PGPS activity are present in mutants lacking mitochondrial DNA (rho0 mutants) compared with isogenic rho+ strains. To determine whether decreased PGPS activity in rho0 mutants can be explained by decreased PGS1 mRNA, mRNA abundance was compared in rho0 and rho+ cells that were isogenic with respect to nuclear DNA. In synthetic glucose medium, PGS1 mRNA was fully derepressed in the rho0 mutant (Fig. 8B). Therefore, the decrease in PGPS activity in the rho0 mutant is not because of decreased PGS1 expression.

PGPS activity is increased 2–3-fold in non-fermentable versus fermentable medium (14). PGS1 mRNA levels were measured in wild-type cells grown in the presence of glucose or glycerol/ethanol as the sole carbon source (Fig. 9B). In the mid-logarithmic growth phase, PGS1 mRNA levels were about 2-fold higher in glycerol/ethanol. However, glucose-grown cells exhibited an increase in PGS1 as cells entered the stationary phase, at which point PGS1 mRNA abundance was higher in glucose than in glycerol/ethanol.

**DISCUSSION**

In this report, we used Northern analysis to look directly at the mRNA abundance of the PGS1 gene in response to regulatory stimuli and concluded the following points. Regulation of PGPS activity by inositol does not occur at the level of mRNA abundance and is not mediated by the INO2-IN4-OPI1 genetic regulatory circuit. Therefore, cross-pathway control of general and mitochondria-specific phospholipid pathways oc-
curs by different regulatory mechanisms. In glucose, PGS1 expression increases during the stationary growth phase. In the logarithmic phase, the increase in PGPS in glycerol/ethanol versus glucose is accompanied by an increase in PGS1 mRNA. However, the decrease in PGPS activity in the absence of a mitochondrial genome is not accompanied by a decrease in PGS1 mRNA. Therefore, PGS1 expression is controlled by the mitochondrial development factors including growth phase and carbon source. Loss of mitochondrial genome, which does lead to decreased PGPS activity, is unaccompanied by altered PGS1 expression.

Our finding that regulation of PGPS by inositol is not via transcriptional control of PGS1 by INO2-INO4-OP1 is consistent with the results of a previous study in which PGPS enzyme activity was measured in response to inositol supplementation (13). Specifically, that PGS1 expression is fully derepressed in ino2 and ino4 null mutants (Fig. 6) is in accord with the finding that PGPS enzyme activity was not altered in these mutants. Similarly, mutation in the OP1 gene did not lead to increased activity of PGPS (13) nor did it affect regulation of PGS1 (Fig. 7). Furthermore, inositol depletion induced by the drug VPA stimulated synthesis of PG without an accompanying increase in PGS1 mRNA abundance (Fig. 5). These results are also consistent with the lack of a functional UASINO element in the promoter region of PGS1 (16). Finally, the increased expression of PGS1 in the stationary phase (Fig. 2) differs from the characteristic increase in expression of UASINO-containing genes during the logarithmic phase of growth. Our findings are not in agreement with results obtained from a study in which PGS1 promoter sequences fused to a reporter construct on an exogenous plasmid appeared to be regulated by inositol (15). In that study, activity of the reporter gene was correlated with expression of native PGS1 mRNA even though it is clear that promoter-driven reporter constructs do not necessarily reflect the regulatory control of a gene in its natural chromosomal context (33). Regulatory sequences may not be present in the construct, and the effects of histone modification and chromatin structure are not necessarily observed with an extrachromosomal reporter. Therefore, it is unlikely that the reporter construct reflected regulation of native PGS1.

PGPS activity responds to mitochondrial development factors such as loss of the mitochondrial genome, carbon source, and growth phase. Loss of the mitochondrial genome did not affect PGS1 expression (Fig. 8), whereas carbon source and growth phase did (Figs. 2 and 9). In this regard, regulation of PGS1 is in concert with expression of the CRD1 gene encoding CL synthase, expression of which increases 7–10-fold in the early stationary phase (34). Therefore, in contrast to the other phospholipids, synthesis of the mitochondrial lipid CL increases during the stationary phase during growth in glucose, a time in which mitochondrial development increases to respond to the switch from fermentative to respiratory growth. In fact, the relative CL composition doubles as cells enter the stationary phase (35, 36). CL may play an important role in cell survival during the stationary growth phase, as both pgs1 and crd1 null mutants lose viability during prolonged liquid culture at elevated temperature (19).3

How PGPS activity is regulated by factors that do not affect PGS1 expression is not known. PGPS activity drops within 20 min following addition of exogenous inositol to the growth medium (13). The rapidity of this response could be attributed

3 Q. Zhong and M. L. Greenberg, unpublished data.
to degradation or inactivation of the protein in the presence of inositol. In a cardiac cell line, stimulation of PGPS activity by 8-(4-chlorophenylthio)-cAMP (CPT-cAMP), a non-hydrolyzable cAMP analogue that activates cAMP-dependent protein kinase, stimulates PGPS activity (37). These findings suggest that phosphorylation may be involved in the regulation of mammalian PGPS. At least one enzyme in general phospholipid synthesis in S. cerevisiae has not been characterized as the general pathway enzymes. Whereas mitochondrial pathway does not involve the same genetic regulatory circuit as the general pathway enzymes. Whereas PGPS activity is decreased in the presence of inositol, regulation of PGSI expression occurs primarily in response to mitochondrial development cues.

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A

B

Fig. 9. Effect of carbon source on PGSI transcription. Wild-type cells (SH302) were grown in synthetic medium with glucose or glycerol/ethanol as the sole carbon source. Cell density (A) was monitored by A520. Cells were harvested at the indicated times, and Northern analysis was performed as described above. Expression of PGSI was normalized by comparison with ACT1. Data represent the average of two independent experiments. [glucose], [glycerol/ethanol].

4 D. Vaden, personal communication.