Regulation of the \textit{DPP1}-encoded Diacylglycerol Pyrophosphate (DGPP) Phosphatase by Inositol and Growth Phase

\textbf{INHIBITION OF DGPP PHOSPHATASE ACTIVITY BY CDP-DIACYLGLYCEROL AND ACTIVATION OF PHOSPHATIDYLSERINE SYNTHASE ACTIVITY BY DGPP*}

Received for publication, September 6, 2000, and in revised form, September 18, 2000
Published, JBC Papers in Press, October 2, 2000, DOI 10.1074/jbc.M008144200

June Oshiro, Shanthi Rangaswamy, Xiaoming Chen, Gil-Soo Han, Jeannette E. Quinn, and George M. Carman†

From the Department of Food Science, Cook College, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, New Jersey 08901

The regulation of the \textit{Saccharomyces cerevisiae} \textit{DPP1}-encoded diacylglycerol pyrophosphate (DGPP) phosphatase by inositol supplementation and growth phase was examined. Addition of inositol to the growth medium resulted in a dose-dependent increase in the level of DGPP phosphatase activity in both exponential and stationary phase cells. Activity was greater in stationary phase cells when compared with exponential phase cells, and the inositol- and growth phase-dependent regulations of DGPP phosphatase were additive. Analyses of DGPP phosphatase mRNA and protein levels, and expression of \(\beta\)-galactosidase activity driven by a \textit{P}_{\text{DPP1-lacZ}} reporter gene, indicated that a transcriptional mechanism was responsible for this regulation. Regulation of DGPP phosphatase by inositol and growth phase occurred in a manner that was opposite of that of many phospholipid biosynthetic enzymes. Regulation of DGPP phosphatase expression by inositol supplementation, but not growth phase, was altered in \textit{opi1Δ}, \textit{ino2Δ}, and \textit{ino1Δ} phospholipid synthesis regulatory mutants. CDP-diacylglycerol, a phospholipid pathway intermediate used for the synthesis of phosphatidylserine and phosphatidylinositol, inhibited DGPP phosphatase activity by a mixed mechanism that caused an increase in \(K_m\) and a decrease in \(V_{\text{max}}\). DGPP stimulated the activity of pure phosphatidylserine synthase by a mechanism that increased the affinity of the enzyme for its substrate CDP-diacylglycerol. Phospholipid composition analysis of a \textit{dpp1Δ} mutant showed that DGPP phosphatase played a role in the regulation of phospholipid metabolism by inositol, as well as regulating the cellular levels of phosphatidylinositol.

The yeast \textit{Saccharomyces cerevisiae} serves as a model eukaryote where the regulation of phospholipid synthesis can be studied (1–4). The major phospholipids found in the membranes of \textit{S. cerevisiae} include PC,\(^1\) PE, PI, and PS (1–4).

\* This work was supported in part by United States Public Health Service Grant GM-28140 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence and reprint requests should be addressed: Dept. of Food Science, Cook College, New Jersey Agricultural Experiment Station, Rutgers University, 65 Dudley Rd., New Brunswick, NJ 08901. Tel.: 732-932-9611 (ext. 217); Fax: 732-932-6776; E-mail: carman@uosop.rutgers.edu.

\(^{1}\) The abbreviations used are: PC, phosphatidylethanolamine; PE, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidate; CDP-DG, CDP-diacylglycerol; DGPP, diacylglycerol pyrophosphate; DG, diacylglycerol; PCR, polymerase chain reaction; kb, kilobase(s); bp, base pair.

Mitochondrial membranes also contain phosphatidylylycerol and cardiolipin (1–4). The synthesis of these phospholipids is a complex process that contains a number of branch points (Fig. 1). PS, PE, and PC are synthesized from PA by the CDP-DG pathway (Fig. 1). CDP-DG is also used for the synthesis of PI and cardiolipin. PE and PC are also synthesized by the CDP-ethanolamine and CDP-choline pathways, respectively (Fig. 1). The CDP-DG pathway is primarily used by wild-type cells for the synthesis of PE and PC when they are grown in the absence of ethanolamine or choline (1, 2, 4–6). The CDP-ethanolamine and CDP-choline pathways assume a critical role in phospholipid synthesis when enzymes in the CDP-DG pathway are defective (1, 2, 4, 7). Mutants in the CDP-DG pathway require choline for growth and synthesize PC by way of CDP-choline (8–15). Mutants defective in the synthesis of PS (8, 9) or PE (10, 11) can also synthesize PC if they are supplemented with ethanolamine. The ethanolamine is used for PE synthesis by way of CDP-ethanolamine. The PE is subsequently methylated to form PC (Fig. 1).

The CDP-ethanolamine and CDP-choline pathways were once viewed as auxiliary or salvage pathways used by cells when the CDP-DG pathway was compromised (1, 2). However, it is now known that these pathways contribute to the synthesis of PE and PC even when wild-type cells are grown in the absence of ethanolamine and choline, respectively (16–18). For example, the PC synthesized via the CDP-DG pathway is constantly hydrolyzed to free choline and PA by phospholipase D (18, 19). Free choline is incorporated back into PC via the CDP-choline pathway, and PA is incorporated into phospholipids via reactions utilizing CDP-DG and DG (2–4) (Fig. 1).

Genetic, molecular, and biochemical studies have shown that the regulation of phospholipid synthesis is a complex and highly coordinated process (2–4). The mechanisms that govern this regulation control the mRNA and protein levels of the biosynthetic enzymes, as well as their activity (1–4). The factors that regulate phospholipid synthesis in \textit{S. cerevisiae} include water-soluble phospholipid precursors, nucleotides, lipids, and growth phase (1, 3, 4, 7, 17).

\textit{DPP1} is a minor phospholipid recently identified in \textit{S. cerevisiae} (20). It contains a pyrophosphatase group attached to DG (21). DGPP is derived from PA via the reaction catalyzed by PA kinase (20). DGPP phosphatase catalyzes the removal of the \(\beta\)-phosphate from DGPP to yield PA and then removes the

\(^{1}\) This paper is available on line at http://www.jbc.org

40887
phosphatase from PA to generate DG (20). The function of DGPP has not been established in S. cerevisiae. However, phospholipid composition analysis of a dpp1Δ mutant devoid of DGPP phosphatase activity (22) has revealed that the DPP1 gene product plays a role in the regulation of phospholipid metabolism (23). The dpp1Δ mutant exhibits a reduction in the cellular level of PI and an elevation in the levels of PA and DGPP.

PA plays a central role in phospholipid synthesis as the upstream phosphatase activity (22) has revealed that the DPP1-encoded DGPP phosphatase. We also discovered that the activity of DGPP phosphatase is inhibited by CDP-DG and that the activity of PS synthase was stimulated by DGPP. The work reported here examined the influence of growth phase on DGPP phosphatase, and we also discovered that the activity of DGPP phosphatase was inhibited by CDP-DG and that the activity of PS synthase was stimulated by DGPP. The phosphatase worked here increased understanding of the long and short term regulation of DGPP phosphatase and the influence of the enzyme on phospholipid metabolism.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were reagent grade. Growth medium supplies were purchased from Difco Radiochemicals were from PerkinElmer Life Sciences. Scintillation counting supplies were from National Diagnostics. Triton X-100, bovine serum albumin, aprotinin, benzamide, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, diethyl pyrocarbonate, inositol, choline, CDP, and O-nitrophenyl β-D-galactopyranoside were purchased from Sigma. Lipids were purchased from Avanti Polar Lipids. DES2 (DEAE-cellulose) was from Whatman. Protein assay reagent, Zeta ProbeTM membranes, electrophoresis reagents, and immunochromogenic reagents were purchased from Bio-Rad. The NEBlot kit, restriction endonuclease modifying enzymes, and recombinant Vent DNA polymerase with 5'- and 3'-exonuclease activity were purchased from New England Biolabs. Primers for polymerase chain reaction were prepared commercially by Genosys Biotechnologies, Inc. Nitrocellulose membranes were purchased from Schleicher & Schuell. ProbeQuant G-50 columns, polyvinylidene difluoride membranes, protein A-Sepharose, and the ECF Western blotting chemifluorescent detection kit were purchased from Amersham Pharmacia Biotech. Silica Gel 60 thin layer chromatography plates were from EM Science.

**Regulation of DPP1-encoded DGPP Phosphatase**

**FIG. 1. Pathways for the synthesis of the major phospholipids in S. cerevisiae.** The pathways shown for phospholipid synthesis include the relevant steps discussed in the text. The four major phospholipids (PC, PE, PI, and PS) are indicated by boxes. The CDP-DG, CDP-choline, and CDP-ethanolamine pathways are indicated. DGPP is indicated by the ellipse. The DPP1-encoded DGPP phosphatase, CHO1-encoded PS synthase, and INO1-encoded inositol-1-P synthase reactions are indicated in the figure. A more comprehensive description that includes the synthesis of PA and additional steps in these pathways can be found elsewhere (2, 17). The abbreviations used are: FMR, phosphatidylmonomethylglycerol; PDE, phosphatidylglycerol; TG, triacylglycerol; PGP, phosphatidylglycerol phosphate; CL, cardiolipin.
A 1.8-kb insert, containing the CHO1 gene fused to the GAL7 promoter, was released from plasmid YCpGPSS (32) by digestion with SalI/BamHI. This DNA fragment was ligated into the SalI/BamHI sites of pRS425, a multicopy E. coli yeast shuttle vector containing the LEU2 gene (33) to form plasmid YEpGPSS. This construct was transformed into W303-1A for the overexpression of the CHO1-encoded PS synthase.

RNA Isolation and Northern Blot Analysis—Total yeast RNA was isolated using the methods of Schmitt et al. (34) and Herrick et al. (35). Equal amounts (25 μg) of total RNA from each sample were resolved on a 1.1% formaldehyde gel for 2.5 h at 100 V (36). The RNA samples were then transferred to a Zeta Probe™ membrane by vacuum blotting.

Pre-hybridization, hybridization with a specific probe, and washes to remove unbound probe were carried out according to the manufacturer’s instructions. The DPP1 probe was a 0.87-kb fragment isolated from pDT1-DPP1 by MfeI/BamHI digestion. A TCOR1 probe was used as a constitutive standard and a loading control. This probe was generated enzymatically using purified DGPP phosphatase and protein in a total volume of 0.1 ml.

Preparations of Enzymes—Cells from all strains were disrupted with glass beads (40) in 50 mM Tris maleate buffer, pH 7.0, containing 1 mM Na₂EDTA, 0.3 mM sucrose, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 5 μg/ml each of aprotinin, leupeptin, and pepstatin. Glass beads and unbroken cells were removed by centrifugation at 1,500 × g for 10 min. The supernatant (cell extract) was used for enzyme assays and immunoblot analysis.

Preparations of Enzymes—Cells from all strains were disrupted with glass beads (40) in 50 mM Tris maleate buffer, pH 7.0, containing 1 mM Na₂EDTA, 0.3 mM sucrose, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 5 μg/ml each of aprotinin, leupeptin, and pepstatin. Glass beads and unbroken cells were removed by centrifugation at 1,500 × g for 10 min. The supernatant (cell extract) was used for enzyme assays and immunoblot analysis.

Preparations of Anti-DGPP Phosphatase Antibodies and Immunoblotting—The peptide sequence SDVTLEEAVTHQRIPDE (residues 263–279 at the C-terminal end of the deduced protein sequence of DPP1) was synthesized and conjugated to carrier protein at Bio-Synthesis, Inc. (Lewisville, TX). Antibodies were raised against the peptide in New Zealand White rabbits by standard procedures (37) at Bio-Synthesis, Inc. The IgG fraction was isolated from antisera by protein A-Sepharose chromatography (37). SDS-polyacrylamide gel electrophoresis (38) using 12% slab gels and immunoblotting (39) using polyvinylidene difluoride membranes were performed as described previously.

The DGPP phosphatase antibodies were used at a dilution of 1:1,000, and the DGPP phosphatase protein was detected using the ECF Western blotting chemiluminescent detection kit as described by the manufacturer.

The DGPP phosphatase protein on immunoblots was acquired by FluorImager or a Typhoon phosphorimager. Immunoblot signals were in the linear range of detectability.

Preparation of Triton X-100/Phospholipid Mixed Micelles—Phospholipids in chloroform were transferred to a test tube, and solvent was removed in vacuo for 40 min. The surface concentration of phospholipids in Triton X-100/phospholipid mixed micelles was varied by the addition of various amounts of an 80 mM solution of Triton X-100 to the dried phospholipids. The total phospholipid concentration in the mixed micelles did not exceed 15 mol % to ensure that the structure of the micelles was similar to that of pure Triton X-100 (44, 45). The mol % of a phospholipid in a mixed micelle was calculated with the following formula: mol % phospholipid = [(mol phospholipid (mm))/[mol phospholipid (mm)] + [Triton X-100 (mm)]] × 100.

Preparation of Anti-DGPP Phosphatase Antibodies and Immunoblotting—The peptide sequence SDVTLEEAVTHQRIPDE (residues 263–279 at the C-terminal end of the deduced protein sequence of DPP1) was synthesized and conjugated to carrier protein at Bio-Synthesis, Inc. (Lewisville, TX). Antibodies were raised against the peptide in New Zealand White rabbits by standard procedures (37) at Bio-Synthesis, Inc. The IgG fraction was isolated from antisera by protein A-Sepharose chromatography (37). SDS-polyacrylamide gel electrophoresis (38) using 12% slab gels and immunoblotting (39) using polyvinylidene difluoride membranes were performed as described previously.

The DGPP phosphatase antibodies were used at a dilution of 1:1,000, and the DGPP phosphatase protein was detected using the ECF Western blotting chemiluminescent detection kit as described by the manufacturer.

The DGPP phosphatase protein on immunoblots was acquired by FluorImager or a Typhoon phosphorimager. Immunoblot signals were in the linear range of detectability.

Preparation of Anti-DGPP Phosphatase Antibodies and Immunoblotting—The peptide sequence SDVTLEEAVTHQRIPDE (residues 263–279 at the C-terminal end of the deduced protein sequence of DPP1) was synthesized and conjugated to carrier protein at Bio-Synthesis, Inc. (Lewisville, TX). Antibodies were raised against the peptide in New Zealand White rabbits by standard procedures (37) at Bio-Synthesis, Inc. The IgG fraction was isolated from antisera by protein A-Sepharose chromatography (37). SDS-polyacrylamide gel electrophoresis (38) using 12% slab gels and immunoblotting (39) using polyvinylidene difluoride membranes were performed as described previously.

The DGPP phosphatase antibodies were used at a dilution of 1:1,000, and the DGPP phosphatase protein was detected using the ECF Western blotting chemiluminescent detection kit as described by the manufacturer.

The DGPP phosphatase protein on immunoblots was acquired by FluorImager or a Typhoon phosphorimager. Immunoblot signals were in the linear range of detectability.

Preparation of Triton X-100/Phospholipid Mixed Micelles—Phospholipids in chloroform were transferred to a test tube, and solvent was removed in vacuo for 40 min. The surface concentration of phospholipids in Triton X-100/phospholipid mixed micelles was varied by the addition of various amounts of an 80 mM solution of Triton X-100 to the dried phospholipids. The total phospholipid concentration in the mixed micelles did not exceed 15 mol % to ensure that the structure of the micelles was similar to that of pure Triton X-100 (44, 45). The mol % of a phospholipid in a mixed micelle was calculated with the following formula: mol % phospholipid = [(mol phospholipid (mm))/[mol phospholipid (mm)] + [Triton X-100 (mm)]] × 100.

Enzyme Assays, Protein Determination, and Analysis of Kinetic Data—DGPP phosphatase activity was measured by following the release of water-soluble [32P]P from chloroform-soluble [β-32P]DGPP (10,000–15,000 cpm/nmol) as described by Wu et al. (20). The reaction mixture contained 50 mM citrate buffer, pH 5.0, 2 mM Triton X-100, 0.1 mM DGPP, and enzyme protein in a total volume of 0.1 ml. β-Galactosidase activity was determined by measuring the conversion of O-nitrophenyl β-D-galactopyranoside to O-nitrophenol (molar extinction coefficient of 3,500 M⁻¹ cm⁻¹) by following the increase in absorbance at 410 nm on a recording spectrophotometer (46). The reaction mixture contained 100 mM sodium phosphate buffer, pH 7.0, 3 mM O-nitrophenyl β-D-galactopyranoside, 1 mM MgCl₂, 100 mM 2-mercaptoethanol, and enzyme protein in a total volume of 0.1 ml. PS synthase activity was measured by following the incorporation of water-soluble [3-H]serine (10,000–40,000 cpm/nmol) into chloroform-soluble PS as described by Bae-Lee and Carman (41). The reaction mixture contained 50 mM Tris-HCl buffer, pH 8.0, 10 mM MgCl₂, 3.2 mM Triton X-100, 0.2 mM CDP-diacylglycerol, 0.5 mM serine, and enzyme protein in a total volume of 0.1 ml. MgCl₂ was used instead of MnCl₂ (the alternative cofactor (41)) because DGPP chelates manganese ions. DGPP phosphatase and β-galactosidase assays were conducted at 30 and 25 °C, respectively. The average standard deviation of the enzyme assays (performed in triplicate) was ± 5%. The enzyme reactions were linear with time and protein concentration. A unit of enzymatic activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product/min unless otherwise indicated. Specific activity was defined as units/mg protein. Protein concentration was determined by the method of Bradford (47) using bovine serum albumin as the standard.

Kinetic data were analyzed with the EZ-FIT enzyme kinetic model-fitting program according to the Michaelis-Menten and Hill equations. EZ-FIT uses the Nelder-Mead Simplex and Marquardt/Nash nonlinear regression algorithms sequentially and tests for the best fit of the data among different kinetic models (48). IC₅₀ values were calculated from plots of the log of activity versus the inhibitor concentration.

Labeling and Analysis of Phospholipids—Labeling of phospholipids with [32P]P was performed as described previously (8, 9, 49). Lipids were extracted from labeled cells by the method of Bligh and Dyer (50) as described previously (51). Phospholipids were separated by DEAE-cellulose chromatography followed by one-dimensional thin layer chromatography on silica gel plates (52). DEAE-cellulose (acetate form)
The amount of the DGPP phosphatase protein was elevated (3-fold) in stationary phase cells grown without inositol. During the course of these experiments, we noted that the total RNA derived from stationary phase cells was especially subject to degradation during its isolation. Thus, it was difficult to quantify the levels of mRNA.

The levels of the DGPP phosphatase protein in response to inositol supplementation and growth phase were examined. Antibodies were generated against a peptide sequence found at the C-terminal end of the DGPP phosphatase protein. These antibodies recognized purified DGPP phosphatase (data not shown), as well as the enzyme in cell extracts. DGPP phosphatase migrates on SDS-polyacrylamide gels as a doublet with a molecular mass of ~34 kDa (20, 55). Immunoblot analysis using these antibodies showed that the levels of the DGPP phosphatase protein were elevated (4- and 3-fold, respectively) in response to inositol supplementation in both the exponential (Fig. 3A) and stationary (Fig. 3C) phase cells. This analysis also showed that the relative amount of DPP1 mRNA in stationary phase cells supplemented with inositol was greater than that found in exponential phase cells grown without inositol. The expression of TCM1 mRNA was also determined and served as a loading control. The TCM1 gene encodes a ribosomal protein that is not regulated by inositol supplementation (53, 54). This analysis showed that the presence of inositol in the growth medium resulted in an increase in the relative amount of DPP1 mRNA in both exponential (Fig. 3A) and stationary (Fig. 3C) phase cells.

The expression of TCM1 mRNA was also determined and served as a loading control. The TCM1 gene encodes a ribosomal protein that is not regulated by inositol supplementation (53, 54). This analysis showed that the presence of inositol in the growth medium resulted in an increase in the relative amount of DPP1 mRNA in both exponential (Fig. 3A) and stationary (Fig. 3C) phase cells. This analysis also showed that the relative amount of DPP1 mRNA in stationary phase cells supplemented with inositol was greater than that found in exponential phase cells grown without inositol. During the course of these experiments, we noted that the total RNA derived from stationary phase cells was especially subject to degradation during its isolation. Thus, it was difficult to quantify the levels of mRNA.

The levels of the DGPP phosphatase protein in response to inositol supplementation and growth phase were examined. Antibodies were generated against a peptide sequence found at the C-terminal end of the DGPP phosphatase protein. These antibodies recognized purified DGPP phosphatase (data not shown), as well as the enzyme in cell extracts. DGPP phosphatase migrates on SDS-polyacrylamide gels as a doublet with a molecular mass of ~34 kDa (20, 55). Immunoblot analysis using these antibodies showed that the levels of the DGPP phosphatase protein were elevated (4- and 3-fold, respectively) in response to inositol supplementation in both the exponential (Fig. 3B) and stationary (Fig. 3D) phases of growth. This analysis also showed that in cells grown without inositol, the level of the DGPP phosphatase protein was elevated (3-fold) in stationary phase when compared with the exponential phase of growth. The amount of the DGPP phosphatase protein in stationary phase cells supplemented with inositol was 7-fold greater when compared with that of exponential phase cells grown without inositol. These results were consistent with the
We utilized a stationary phase of growth. DGPP phosphatase mRNA and protein in the exponential and stationary phases of growth. The abundance of DPP1 mRNA was determined by Northern blot analysis. 25 μg of total RNA was applied to each lane. Portions of Northern blots are shown, and the positions of DPP1 mRNA and TCM1 mRNA (loading control) are indicated. Cell extracts were prepared from cells harvested in the exponential (A) and stationary (C) phases of growth. These results were consistent with the data on DGPP phosphatase activity (Fig. 2). The addition of inositol to the growth medium resulted in a dose-dependent increase in β-galactosidase activity (Fig. 4). The level of activity in cell extracts derived from cells supplemented with 40–50 μM inositol was 2.6-fold greater than the activity from cells grown in the absence of inositol (Fig. 4). The addition of inositol to cells at concentrations greater than 50 μM inositol resulted in a dose-dependent decrease in β-galactosidase activity (Fig. 4). These results were consistent with the data on DGPP phosphatase activity (Fig. 2A).

The β-galactosidase activity, driven by the P_{DPP1}–lacZ reporter gene, was also measured in stationary phase cells grown in the absence and presence of 50 μM inositol (Fig. 5C). In the absence of inositol supplementation, the β-galactosidase activity in stationary phase cells was 4.6-fold greater than the activity found in exponential phase cells (Fig. 5A). The level of β-galactosidase activity in inositol-supplemented stationary phase cells was 1.8-fold greater when compared with stationary phase cells grown without inositol (Fig. 5C). The specific activity of β-galactosidase in stationary phase cells supplemented with inositol (Fig. 5C) was 8.5-fold greater than that of exponential phase cells grown with inositol (Fig. 5A). These data further confirmed that a transcriptional mechanism was responsible for the changes in DGPP phosphatase activity (Fig. 5).

For many phospholipid biosynthetic enzymes, the repressive effect of inositol is enhanced by the inclusion of 1 mM choline to the growth medium (1, 2, 4, 7). We examined whether choline alone or in combination with inositol affected the expression of the DPP1 gene and DGPP phosphatase activity. This analysis showed that choline supplementation had no effect on the regulation of DGPP phosphatase (data not shown). The effect of inositol is enhanced by the inclusion of 1 mM choline to the growth medium (1, 2, 4, 7). For example, Opi1p represses the expression of INO1-encoded inositol-1-P synthase and CHO1-encoded PS synthase, whereas Ino2p and Ino4p induce the expression of these enzymes (40, 54, 56–65). An opi1 mutant exhibits elevated expression of phospholipid biosynthetic enzymes, and the expression of these enzymes does not respond to inositol supplementation (1, 7). Owing to the constitutive low expression of the INO1 gene (53), ino2 and ino4 mutants are inositol auxotrophs (28). These mutants also exhibit repressed levels of the phospholipid biosynthetic enzymes that are repressed by inositol (1, 7, 17, 66). Moreover, the expression of the inositol-regulated enzymes in ino2 and ino4 mutants is not affected by inositol supplementation (1, 7, 17, 66). For cells grown to exponential phase without inositol, the expression of P_{DPP1}–lacZ driven β-galactosidase activity was 2-fold lower in opi1Δ mutant cells when compared with wild-type cells (Fig. 5A). In contrast to wild-type cells, the addition of inositol to the growth medium of opi1Δ cells did not result in an increase in DPP1 expression. Instead, inositol supplementation caused a small decrease in DPP1 expression. We next examined the expression of DGPP phosphatase activity in exponential phase opi1Δ cells (Fig. 5B). The DGPP phosphatase activity in cells grown in the absence of inositol was slightly higher than that found in wild-type cells. This level of DGPP phosphatase activity did not correlate with the reduced level of β-galactosidase activity in opi1Δ cells when compared with the wild-type control (Fig. 5A). Whether this difference was due to transcriptional control versus translational control will require additional studies. The addition of inositol to opi1Δ cells resulted in a small reduction in DGPP phosphatase activi-
Phosphatase regulation by growth phase were examined. The was reduced in the exponential phase of growth when compared with the activities in exponential phase (2.7- and 1.6-fold, respectively) in wild-type cells. Like exponential phase 

opi1

mutant cells, the expression of 

b

mutant cells when compared with these activities in exponential phase cells. However, in contrast to the lower activity found in the exponential phase, the level of expression of 

b

mutant cells in stationary phase 

opi1

cells was similar to the 

b

mutant cells expression found in wild-type stationary phase cells. Like exponential phase 

opi1

mutant cells, the expression of 

b

mutant cells and DGPP phosphatase activities did not increase in response to inositol supplementation in stationary phase (Fig. 5, C and D).

The regulation of DGPP phosphatase was examined in 

ino2Δ

and 

ino4Δ

mutant cells grown in the presence of 10 and 60 μM inositol. The 10 μM concentration of inositol was required to support the growth of these inositol auxotrophs (68). This growth condition was considered analogous to the growth condition of wild-type cells grown in the absence of inositol (53). The expression of 

b

mutant cells in exponential phase was reduced when compared with that found in wild-type cells (Fig. 5A). The DGPP phosphatase activity in exponential phase 

ino2Δ

and 

ino4Δ

mutant cells was similar to that of wild-type cells (Fig. 5B). The addition of 60 μM inositol to the growth medium had small effects on the expression of both 

b

mutant cells (Fig. 5A) and DGPP phosphatase (Fig. 5B) activities in the 

ino2Δ

and 

ino4Δ

mutant cells.

The effects of the 

ino2Δ

and 

ino4Δ

mutations on DGPP phosphatase regulation by growth phase were examined. The 

b

mutant cells were elevated when compared with these activities from exponential phase 

ino2A

and 

ino4A

mutant cells. However, both 

b

mutant cells and DGPP phosphatase activities were lower (2.7- and 1.6-fold, respectively) in 

ino2A

and 

ino4A

mutant cells when compared with wild-type cells. The 

b

mutant cells phosphatase activities in the 

ino4Δ

mutant were similar to the levels of these activities found in wild-type cells (Fig. 5, C and D). The expression of 

b

mutant cells (Fig. 5, C and D). The expression of 

b

mutant cells and DGPP phosphatase activities in stationary phase 

ino2A

and 

ino4A

mutant cells supplemented with 60 μM inositol were elevated but not to the levels observed in wild-type cell supplemented with inositol (Fig. 5, C and D).

**Effect of the dpp1Δ Mutation on the Regulation of the INO1 Gene by Inositol and Growth Phase**—We questioned whether the 

dpp1Δ

mutant cells. However, both 

b

mutant cells did not have a major effect on this regulation (data not shown).

**Inhibition of DGPP Phosphatase Activity by CDP-DG**—We initiated studies to examine the regulation of DGPP phosphatase activity on a biochemical level. Being a membrane-associated enzyme (20, 22), DGPP phosphatase has a distinct relationship with its neighboring phospholipids. Thus, we examined whether phospholipids played a role in the biochemical regulation of the enzyme. For these studies, we used purified DGPP phosphatase and Triton X-100/phospholipid mixed micelles so the effects of phospholipids on activity could be examined under well defined conditions. The nonionic detergent Triton X-100 is required to elicit a maximum turnover for DGPP phosphatase activity in vitro (20). The function of Triton X-100 in the assay system for DGPP phosphatase is to form a uniform mixed micelle with the substrate DGPP (20). The Triton X-100 micelle serves as a catalytically inert matrix in which the DGPP is dispersed, preventing a high local concentration of substrate at the active site (71). In addition, this micelle system permitted the analysis of DGPP phosphatase

**FIG. 5. Effects of the op1Δ, ino2Δ, and ino4Δ mutations on the regulation of DGPP phosphatase by inositol and growth phase.** Wild-type, 

op1Δ, 

ino2Δ, and 

ino4Δ

cells bearing the 

Pino2

lacZ

reporter plasmid pJO2 were grown in the absence and presence of 50 μM inositol. Cultures were harvested in the exponential and stationary phases of growth. Cell extracts were prepared and assayed for 

b-galactosidase activity (A and C) and for DGPP phosphatase activity (B and D). Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D. WT, wild-type.
activity in an environment that mimics the physiological surface of the membrane (71). In Triton X-100/phospholipid-mixed micelles, DGPP phosphatase activity follows surface dilution kinetics (71), where activity is dependent on both the molar and surface concentrations of DGPP (20). In the experiments reported here, DGPP phosphatase activity was measured such that activity was only dependent on the surface concentration of DGPP (20). The concentrations of DGPP and other phospholipids were expressed as a surface concentration (in mol %), as opposed to a molar concentration, since phospholipids form uniform mixed micelles with Triton X-100 (44, 45).

DGPP phosphatase activity was assayed in the presence of various phospholipids. The major yeast phospholipids PC, PE, PI, and PS inhibited DGPP phosphatase activity at a final concentration of 10 mol % (Fig. 7A). However, these phospholipids were not considered to be strong inhibitors (30% or less) and were not pursued further. PA, at concentrations up to 10 mol %, does not affect DGPP phosphatase activity (20). On the other hand, the addition of CDP-DG to the assay system resulted in a dose-dependent inhibition (IC50 = 5.3 mol %) of DGPP phosphatase activity (Fig. 6A). DGPP phosphatase activity was measured in the presence of CDP and DG to examine which part of the CDP-DG molecule was responsible for the inhibition. CDP caused a dose-dependent inhibition (IC50 = 2.6 mM) of activity (Fig. 6B). DG caused a small increase (25%) in DGPP phosphatase activity at a final concentration of 4 mol % (Fig. 6A). These data indicated that the inhibition by CDP-DG may be attributed to the CDP moiety of the molecule. A kinetic analysis was performed on DGPP phosphatase to explore the mechanism of inhibition by CDP-DG. The dependence of DGPP phosphatase activity on DGPP was examined in the absence and presence of 4 mol % CDP-DG (Fig. 6C). As described previously (20), DGPP phosphatase exhibited saturation kinetics with respect to the surface concentration of DGPP. CDP-DG inhibited DGPP phosphatase activity in a dose-dependent manner at each DGPP concentration. An analysis of the kinetic data showed that CDP-DG was a mixed type of inhibitor (72) causing an increase in K_m and a decrease in V_max. The K_i value for CDP-DG was calculated to be 5 mol %.

Activation of PS Synthase Activity by DGPP—PS synthase is one of the most highly regulated enzymes of phospholipid metabolism (1, 2, 7, 17), and the biochemical regulation of the purified enzyme (41) has been extensively studied (3, 4). In light of the fact that CDP-DG regulated DGPP phosphatase activity, we examined the effect of DGPP on PS synthase activity. For these studies, we utilized pure enzyme and the Triton X-100/phospholipid mixed micelle system as discussed above for DGPP phosphatase. The addition of DGPP to the assay system resulted in a dose-dependent stimulation of PS synthase activity (Fig. 7A). The concentration of DGPP that resulted in half-maximum activation (A_0.5) was 0.13 mol %. The stimulation by DGPP could be attributed to the pyrophosphate moiety of the molecule since DG does not stimulate PS synthase activity (73). At high concentrations (e.g. 11 mol %), DG
The indicated *S. cerevisiae* strains were grown to the stationary (1 × 10⁶ cells/ml) phase of growth in complete synthetic medium in the absence and presence of 50 μM inositol. The steady-state phospholipid composition was determined by labeling cells for 12 generations with 32P (5 μCi/ml). The incorporation of 32P into phospholipids during the labeling was approximately 2400 cpm/10⁶ cells. The phospholipid composition of the cells was determined as described under “Experimental Procedures.” The percentages shown for phospholipids were normalized to the total 32P-labeled chloroform-soluble fraction that included sphingolipids and other unidentified phospholipids. The values reported are the average of two independent experiments.

| Growth condition | PC | PE | PI | PS | PA | DGPP | CDP-DG | PI:PC |
|------------------|----|----|----|----|----|------|--------|-------|
| Wild type        | 38 ± 1.0 | 10 ± 3.0 | 4.0 ± 0.3 | 3.8 ± 0.05 | 1.2 ± 0.3 | 0.4 ± 0.1 | 0.20 ± 0.1 | 0.11   |
| *dpp1Δ*          | 39 ± 1.4 | 12 ± 0.6 | 3.5 ± 0.2 | 2.4 ± 0.03 | 0.9 ± 0.08 | 0.4 ± 0.2 | 0.37 ± 0.08 | 0.09   |
| + 50 μM inositol | Wild type | 35 ± 2.3 | 12.1 ± 1.2 | 18 ± 1.0 | 3.9 ± 0.6 | 1.2 ± 0.1 | 0.4 ± 0.2 | 0.26 ± 0.1 | 0.51   |
| *dpp1Δ*          | 39 ± 1.1 | 12.8 ± 1.6 | 4.8 ± 0.1 | 4.0 ± 0.8 | 1.1 ± 0.04 | 0.3 ± 0.01 | 0.38 ± 0.1 | 0.12   |

Effect of a *dpp1Δ* Mutation on the Phospholipid Composition of Stationary Phase Cells Grown in the Presence of Inositol—We examined the effects of a *dpp1Δ* mutation on the phospholipid composition of stationary phase cells. Stationary phase cells that were grown in the absence and presence of 50 μM inositol were labeled with 32P. Phospholipids were extracted and analyzed as described under “Experimental Procedures.” When grown without inositol supplementation, the amounts of the major phospholipids (i.e., PC, PE, PI, and PS) as well as PA and DGPP were not significantly affected by the *dpp1Δ* mutation (Table II). The amount of CDP-DG in the *dpp1Δ* mutant was nearly 50% greater than that of wild-type cells grown without inositol (Table II). The presence of inositol in the growth medium of wild-type cells resulted in a 4.5-fold increase in PI content and a small increase in CDP-DG content (Table II). On the other hand, the PI content in *dpp1Δ* mutant cells increased by only 1.37-fold, and the CDP-DG content did not change when cells were supplemented with inositol (Table II). The PI:PC ratio in the *dpp1Δ* mutant grown in the presence of inositol was 4.2-fold lower than that of wild-type cells (Table II). The amounts of the other phospholipids in the *dpp1Δ* mutant supplemented with inositol were not significantly different from that of wild-type cells supplemented with inositol.

**DISCUSSION**

The identification and initial analysis of *DPP1*-encoded DGPP phosphatase indicates that this enzyme plays a previously unidentified role in phospholipid metabolism in *S. cerevisiae* (20, 22). It has been postulated that DGPP may function in a novel lipid-signaling pathway (4, 74). Studies with plants, where DGPP was first discovered (21), have shown that this phospholipid accumulates upon G protein activation (75) or hyperosmotic stress (76, 77). DGPP accumulation is transient (77), and it is rapidly converted to PA and then to DG (42). In *S. cerevisiae*, DGPP phosphatase may function to regulate cellular levels of DGPP, PA, and DG (3, 4). DGPP has not been identified in mammalian cells. However, Balboa et al. (78) have shown that exogenous DGPP activates mouse macrophages for enhanced secretion of arachidonic acid metabolites, a key event in the immunoinflammatory response of leukocytes.

To gain insight into the role that DGPP phosphatase plays in *S. cerevisiae*, we examined the effects of inositol supplementation and growth phase on the expression of the enzyme. These two growth conditions have a major impact on the expression of several phospholipid biosynthetic enzymes (1, 2, 4, 7, 17). Inositol supplementation to wild-type cells resulted in the elevation of DGPP phosphatase activity in both the exponential and stationary phases of growth. DGPP phosphatase activity was higher in stationary phase cells when compared with exponential phase cells. Moreover, the inositol- and growth phase-dependent regulation of the enzyme were additive. Analyses of the DGPP phosphatase mRNA abundance and protein levels, as well as the expression of β-galactosidase activity driven by a *P_DPP1-lacZ* reporter gene, showed that a transcriptional mechanism was responsible for this regulation. The effects of inositol and growth phase on DGPP phosphatase expression were opposite that of most phospholipid biosynthetic enzymes. For example, CDP-DG pathway enzymes (i.e., CDP-DG synthase, PS synthase, PS decarboxylase, and phospholipid N-methyltransferases) are repressed when wild-type cells are supplemented with inositol and when they enter the stationary phase of growth (1, 2, 4, 7, 17). The repression of these enzymes by inositol supplementation or in stationary phase is mediated by a UASINO cis-acting element (1, 64, 79) present in the promoters of their genes (1, 2, 4, 7, 17). The promoter of the *DPP1* gene does not contain a UASINO element. Additional studies will be required to identify the promoter element(s) responsible for the inositol- and growth phase-dependent regulation of the *DPP1* gene.

DGPP phosphatase is not the first example of an enzyme that is regulated by inositol or by growth phase in a manner that is opposite to the co-regulated phospholipid biosynthetic enzymes whose genes contain a UASINO element (4). These enzymes include 45-kDa Mg²⁺-dependent PA phosphatase (51, 80) and inositol-1-P phosphatase (81, 82). The magnitude of the regulation by inositol supplementation and growth phase observed for DGPP phosphatase was significantly greater than that of these other enzymes. Expression of cardiolipin synthase
is not regulated by inositol supplementation, but it is derepressed in stationary phase (85). Phosphatidylglycerophosphate synthase, whose promoter contains a UASINO element, is repressed by inositol supplementation (84, 85) but is derepressed in the stationary phase (86).

The UASINO element contains the binding site for the Ino2p-Ino4p complex, which is necessary for maximum expression of the co-regulated UASINO-containing genes (4, 7, 17, 53, 67). Repression of the co-regulated genes requires the transcription factor Opilp (59, 87). Although Opilp functions via the UASINO element (63), it does not bind the element directly and does not interact with Ino2p or Ino4p (88). The DPP1 gene does not have a UASINO element, yet Opilp, Ino2p, and Ino4p played a role in its regulation by inositol. In both exponential and stationary phase cells, expression of DPP1 was not regulated normally by inositol in the opilΔ, ino2Δ, and ino4Δ mutants. Moreover, the aberrant regulation observed in the ino2Δ and ino4Δ mutants differed from each other (expression of DPP1 was greater in the ino4Δ mutant). This suggests that an Ino2p-Ino4p complex may be required for proper DPP1 regulation. Differential regulatory effects between the ino2Δ and ino4Δ mutants have been observed for the 45-kDa Mg2+-dependent PA phosphatase (51), PI synthase (89), and for cardiolipin synthase.3 The regulation of DPP1 by growth phase was not affected in the regulatory mutants suggesting that Opilp, Ino2p, and Ino4p do not play a role in the growth phase-dependent regulation of DGPP phosphatase.

Phospholipid synthesis in S. cerevisiae has been described as existing in two regulatory states designated as “on” and “off” (4). The system is on when the UASINO-containing genes are maximally expressed and off when they are repressed. When the UASINO-containing genes are on, the DPP1 gene is off and vice versa. Analysis of phenotypes associated with mutations in the UASINO-containing genes have led to the hypothesis that PA, or a closely related metabolite, is responsible for generating a signal that causes the derepression of these genes (4, 17). According to the model, the system is on when PA is produced more rapidly than it is consumed, and the system is off when PA is consumed more rapidly than it is produced (4). Since DGPP phosphatase is involved in the metabolism of PA, we questioned whether the enzyme was involved in the regulation of the UASINO-containing genes. The dpp1Δ mutant did not have a significant effect on the regulation of the INO1 gene by inositol. If DGPP phosphatase were involved, other enzymes (e.g. PA kinase, CDP-DG synthase, and phospholipase D) that catalyze reactions affecting the metabolism of PA may have compensated for the dpp1Δ mutation. A mutation in the LPFP1 gene, which encodes a lipid phosphatase that utilizes PA and DGPP as substrates (23, 90), does not affect the regulation of the INO1 gene.4

CDP-DG and DGPP, substrates of PS synthase and DGPP phosphatase, regulated DGPP phosphatase and PS synthase activities, respectively. CDP-DG was a mixed type inhibitor of DGPP phosphatase. The inhibitor constant for CDP-DG (KΙ = 5 mol %) was about 12-fold higher than its cellular concentration in stationary phase cells (Table II). On the other hand, the KΙ value was within the range of the cellular concentration of CDP-DG in exponential phase cells (2–11 mol %) (91, 92). Thus, it is unlikely that CDP-DG regulates DGPP phosphatase activity in stationary phase cells, but regulation of the phosphatase by CDP-DG may be physiologically relevant in exponential phase cells. DGPP stimulated PS synthase through a mechanism that involved an increase in the affinity of the enzyme for CDP-DG. The activation constant for DGPP (A>K = 0.13 mol %) was within the range of its cellular concentration in both exponential (0.2–0.4 mol %) (20, 22) and stationary (Table II) phase cells. Thus, regulation of PS synthase activity by DGPP may occur in vivo during both phases of growth.

Previous studies have shown that the regulation of PS synthase is the major factor that controls the synthesis of PS and PI from their common precursor CDP-DG (1, 3, 4). Stimulation of PS synthase by DGPP would favor the synthesis of PS at the expense of PI. DGPP did not affect the activity of PI synthase (data not shown). The partitioning of CDP-DG between PS and PI may be controlled by the activity and/or expression of DGPP phosphatase. For example, reduced levels of DGPP phosphatase would favor the synthesis of PS over PI. Indeed, the major impact of the dpp1Δ mutation in stationary phase cells supplemented with inositol was a decrease in PI content when compared with wild-type cells. Moreover, this caused a major change in the PI:PC ratio, an index of phospholipid synthesis regulation (93, 94).

In summary, the work reported here supported the conclusion that the DPP1-encoded DGPP phosphatase was regulated by genetic and biochemical mechanisms. Moreover, the enzyme played a role in the regulation of phospholipid synthesis by inositol. The synthesis of PI is coordinately regulated with the synthesis of PC by both genetic and biochemical mechanisms (1–3, 7, 17). Clearly, DGPP phosphatase plays a role in this complex regulation.

Acknowledgments—We thank William Dowhan for helpful discussions and for plasmid pSD90. David A. Toke is acknowledged for helpful suggestions with PCR and with the construction of plasmids. We thank Susan A. Henry for providing the INO1 gene.4

REFERENCES

1. Carman, G. M., and Henry, S. A. (1989) Annu. Rev. Biochem. 58, 635–669
2. Paltauf, F., Kohlwein, S. D., and Henry, S. A. (1992) in The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression (Jones, E. W., Pringle, J. R., and Brach, J. R., eds) pp. 415–500, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
3. Carman, G. M., and Zemetra, G. M. (1990) J. Biol. Chem. 265, 13293–13296
4. Carman, G. M., and Henry, S. A. (1999) Proc. Natl. Acad. Sci. 96, 361–369
5. Henry, S. A. (1982) in The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression (Strathern, J. N., Jones, E. W., and Brach, J. R., eds) pp. 101–158, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
6. Carman, G. M. (1989) in Phosphatidycholine Metabolism (Vance, D. E., ed) pp. 165–183, CRC Press, Inc., Boca Raton, FL
7. Greenberg, M. L., and Lopes, J. M. (1996) Microbiol. Rev. 60, 1–20
8. Atkinson, K., Fogel, S., and Henry, S. A. (1980) J. Biol. Chem. 255, 6653–6661
9. Atkinson, K. D., Jensen, B., Kolat, A. I., Storm, E. M., Henry, S. A., and Fogel, S. (1980) J. Bacteriol. 141, 558–564
10. Trotter, P. J., and Voelker, D. R. (1995) J. Biol. Chem. 270, 6062–6070
11. Trotter, P. J., Pedretti, J., Yates, R., and Voelker, D. R. (1995) J. Biol. Chem. 270, 6071–6080
12. Kodaki, T., and Yamashita, S. (1987) J. Biochem. 102, 1542–1543
13. Kodaki, T., and Yamashita, S. (1989) Eur. J. Biochem. 185, 243–251
14. Summers, R. F., Letts, V. A., McGraw, P., and Henry, S. A. (1988) Genetics 120, 969–982
15. McGraw, P., and Henry, S. A. (1989) Genetics 122, 317–330
16. Kim, K., Kim, K.-H., Storey, M. K., Voelker, D. R., and Carman, G. M. (1999) J. Biol. Chem. 274, 14857–14866
17. Henry, S. A., and Patton-Vogt, J. L. (1998) Proc. Natl. Acad. Sci. Res. 61, 133–179
18. Patton-Vogt, J. L., Grieco, P., Sreevathsan, A., Bruno, V., Dowd, S., Swede, M. J., and Henry, S. A. (1997) J. Biol. Chem. 272, 20873–20883
19. Xie, Z. G., Fang, M., Rivaz, M. P., Faulkner, A. J., Sternweis, P. C., Engelbrecht, J., and Bankaitis, V. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12346–12351
20. Wu, W.-I., Liu, Y., Riedel, B., Wissing, J. B., Fischl, A. S., and Carman, G. M. (1999) J. Biol. Chem. 274, 18668–18676
21. Wissing, J. B., and Behrbohm, H. (1993) FEBS Lett. 315, 95–99
22. Toke, D. A., Bennett, W. L., Oshino, J., Wu, W. I., Voelker, D. R., and Carman, G. M. (1999) J. Biol. Chem. 274, 14331–14338
23. Henry, S. A., Atkinson, K. D., Kolat, A. J., and Culbertson, M. R. (1977) J. Bacteriol. 130, 472–484

3 W. Dowhan, personal communication.
4 J. E. Quinn and G. M. Carman, unpublished data.
