Regulation of the Yeast DPP1-encoded Diacylglycerol Pyrophosphate Phosphatase by Transcription Factor Gis1p*

June Oshiro‡§, Gil-Soo Han‡, Wendy M. Iwaynshyn‡, Kristi Conover*, and George M. Carman†‡¶

From the ‡Graduate Program in Microbiology and Molecular Genetics and the ¶Department of Food Science, Rutgers University, New Brunswick, New Jersey 08901

The Saccharomyces cerevisiae DPP1-encoded diacylglycerol pyrophosphate phosphatase catalyzes the dephosphorylation of diacylglycerol pyrophosphate to form phosphatidate and P. The enzyme also dephosphorylates phosphatidate to form diacylglycerol and P. Because diacylglycerol pyrophosphate, phosphatidate, and diacylglycerol have roles as lipid signal molecules in higher eukaryotic cells, it is important to understand how diacylglycerol pyrophosphate phosphatase is regulated. Analysis of DPP1 expression using P<sub>DPPI</sub>-lacZ reporter genes with a series of deletions from the 5′ end of the promoter indicated sequences responsible for enzyme expression. Three binding sites (URS<sub>PP</sub>) for transcription factor Gis1p were identified in the DPP1 promoter (consensus sequence of 5′-T(A/T)AGGGAT-3′). A gis1 mutant exhibited elevated levels of DPP1 expression and diacylglycerol pyrophosphate phosphatase activity. Direct interaction between Gis1p and DPP1 promoter elements was demonstrated by electrophoretic mobility shift assays. Mutations in the three URS<sub>PP</sub> elements within the DPP1 promoter abolished Gis1p-DNA interactions in vitro and abolished the regulation of DPP1 in vivo. These data indicated that Gis1p was a repressor of DPP1 expression. Phospholipid composition analysis of the gis1 mutant showed that Gis1p played a role in regulating the cellular level of diacylglycerol pyrophosphate, as well as the levels of the major phospholipids phosphatidylethanolamine and phosphatidylcholine.

DGPP<sup>1</sup> is a minor phospholipid in the yeast Saccharomyces cerevisiae (1). Its formation from phosphatidate and ATP is catalyzed by phosphatidate kinase (1, 2). Research with plants suggests that DGPP functions as a signal molecule under stress conditions. DGPP accumulates upon hyperosmotic stress (3), dehydration (3), G-protein activation (4), stress conditions. DGPP accumulates upon hyperosmotic stress (3), dehydration (3), G-protein activation (4), stress conditions. For example, in S. cerevisiae, the DPP1-encoded DGPP phosphatase enzyme (1) catalyzes these sequential reactions. The yeast DGPP phosphatase enzyme is a member of the lipid phosphate phosphatase superfamily (10, 11), which contains a three-domain lipid phosphatase motif (12) that is required for catalytic activity (13). DGPP phosphatase utilizes a wide range of lipid phosphate substrates in vitro (1, 14, 15). However, only DGPP and phosphatidate have been shown to be physiologically relevant (16).

To better understand the function of DGPP, we have focused on the regulation of DGPP phosphatase expression in S. cerevisiae. DPP1 expression increases under stressful growth conditions. For example, the expression of DPP1 and DGPP phosphatase activity is induced in stationary phase (17), a condition when cells cease proliferation because of nutrient starvation (18). This regulation is further enhanced by inositol supplementation (17). In addition, DPP1 expression is tightly coupled to zinc homeostasis (19). Zinc deprivation results in induced levels of DPP1 expression and the DGPP phosphatase enzyme (19). In fact, DPP1 is one of the most highly regulated genes that respond to zinc in the entire genome (20, 21).

In this work, we demonstrated that DPP1 was negatively regulated by the transcription factor Gis1p. Gis1p is primarily expressed during stationary phase (22) and functions as both a transcription repressor (23) and activator (22). Our analysis showed that Gis1p repressed the expression of DPP1 and DGPP phosphatase. Gis1p bound to three URS<sub>PP</sub> elements within the DPP1 promoter, and loss of any URS<sub>PP</sub> element affected DPP1 expression in vitro. Phospholipid analysis of a gis1 mutant showed a decrease in the level of DGPP, as well as alterations in the major phospholipids phosphatidylethanolamine and phosphatidylcholine.

**Experimental Procedures**

Materials—All chemicals were reagent grade. Growth medium supplements were purchased from Difco Laboratories. Radiochemicals were from PerkinElmer Life Sciences. Scintillation counting supplies were from National Diagnostics. Bovine serum albumin and goat anti-rabbit alkaline phosphatase-linked IgG antibodies were purchased from Pierce. Triton X-100, aprotinin, benzamidine, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, imidazole, ampicillin, chloramphenicol, and O-nitrophenyl β-D-galactopyranoside were purchased from Sigma. Phospholipid standards for thin layer chromatography were from...
Avanti Polar Lipids, Inc. DNA size ladder for agarose gel electrophoresis, protein size ladder for SDS-PAGE, electrophoresis reagents, protein assay reagent, and PolyPrep columns were purchased from Bio-Rad. Restriction endonucleases, T4 DNA ligase, Klenow Fragment, and re- combinant Vent DNA polymerase with 5'- and 3'-exonuclease activity were purchased from New England Biolabs. Oligonucleotides for PCR reactions and electrophoretic mobility shift assays were prepared commercially by Genosys Biotechnologies, Inc. ProbeQuant G-50 columns and the enhanced chemiluminescence Western blotting detection kit were purchased from Amersham Biosciences. The QuickChange™ site-directed mutagenesis kit was purchased from Stratagene. NIT2-NTA-agarose, QIAprep® Spin Miniprep kit, and QIAquick® gel extraction kit were purchased from Qiagen. The Yeastmaker™ yeast transformation system 2 kit was obtained from Clontech. silica gel 60 thin layer chromatography plates were from EM Science.

**Strains and Growth Conditions**—The strains used in this work are listed in Table I. Methods for yeast growth were performed as described previously (19, 24). Yeast cultures were grown at 30 °C in YPD medium (1% yeast extract, 2% peptone, 2% glucose). Cultures were grown until the cell density reached an A600nm of 0.5. Expression of the GIS1 gene was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside to the growth medium. After incubation for 2 h at 30 °C, cells were harvested by centrifugation at 5,000 × g for 5 min at 4 °C.

**DNA Manipulations and Amplification of DNA by PCR**—Plasmid and genomic DNA preparation, restriction enzyme digestion, and DNA ligations were performed by standard protocols (26). Transformation of E. coli (30) and yeast (27) were performed as described previously. Conditions for the amplification of DNA by PCR were optimized as described by Innis and Gelfand (28). For amplification of DNA by PCR using Vent polymerase as described by Innis and Gelfand (28).

**TABLE I**

| Strain | Relevant characteristics | Source |
|--------|--------------------------|--------|
| S. cerevisiae | MATα leu2-3,112 trp1-1 can1-100 urs3-1 ade2-1 his3-11.15 | Ref. 47 |
| W3031A | MATα leu2-3,112 trp1-1 can1-100 urs3-1 ade2-1 his3-11.15 | This study |
| JOY27 | MATα leu2-3,112 trp1-1 can1-100 urs3-1 ade2-1 his3-11.15 | This study |
| JOY38 | MATα leu2-3,112 trp1-1 can1-100 urs3-1 ade2-1 his3-11.15 | This study |
| E. coli | DH5α | Ref. 26 |
| | BL21(DE3)pLys | Novagen |

**TABLE II**

| Plasmid | Relevant characteristics | Source |
|---------|--------------------------|--------|
| pRS413 | Single copy E. coli/yeast shuttle vector containing HIS3 | Ref. 48 |
| pRS415 | Single copy E. coli/yeast shuttle vector containing LEU2 | Ref. 48 |
| pPCR-Script™ AMP SK(+) | Cloning vector derived from the pBlueScriptII SK(+) phagemid, modified SrfI restriction endonuclease target sequence | Stratagene |
| pJO2 | EcoRI fragment of pJO2 containing the DPP1 promoter ligated into pPCR-Script™ AMP SK(+) | Ref. 17 |
| pJO18 | Derivative of pJO18 containing the DPP1 promoter with a mutation in URS <ref>1</ref> | This study |
| pJO18-S1 | Derivative of pJO18 containing the DPP1 promoter with a mutation in URS <ref>2</ref> | This study |
| pJO18-S2 | Derivative of pJO18 containing the DPP1 promoter with a mutation in URS <ref>3</ref> | This study |
| pJO20 | Derivative of pJO2 containing the DPP1 promoter with a mutation in URS <ref>1</ref> | This study |
| pJO21 | Derivative of pJO2 containing the DPP1 promoter with a mutation in URS <ref>2</ref> | This study |
| pJO22 | Derivative of pJO2 containing the DPP1 promoter with a mutation in URS <ref>3</ref> | This study |
| YEp352 | Multicopy E. coli/yeast shuttle vector containing URA3 | Ref. 29 |
| pJO19 | GIS1 gene ligated into the SacI/SalI site of YEp352 | Novagen |
| pET-15b | E. coli expression vector under T7 lac promoter for N-terminal His-tag fusion | This study |
| pGH305 | GIS1 ORF ligated into the XhoI/BamHI site of pET-15b | This study |

For heterologous expression of the His-tagged GIS1 gene product, E. coli strain BL21(DE3)pLysS bearing plasmid pGH305 was grown in 4 ml of LB medium containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml). Cells were grown to saturation at 37 °C, and the cultures were diluted into 50 ml of fresh medium (initial cell density of A600nm = 0.025). Cultures were grown until the cell density reached an A600nm of 0.5, diluted into 1000 ml of fresh medium, and grown until the cell density reached an A600nm of 0.5. Expression of the GIS1 gene was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside to the growth medium. After incubation for 2 h at 30 °C, cells were harvested by centrifugation at 5,000 × g for 5 min at 4 °C. DNA manipulations and amplification of DNA by PCR were performed using standard procedures as described previously. For amplification of DNA by PCR using Vent polymerase as described by Innis and Gelfand (28). Plasmid maintenance and amplifications were performed in E. coli strain DH5α. DNA sequencing reactions were performed by dye-exchange method using Taq polymerase (26). Plasmid constructions—The plasmids used in this work are listed in Fig. 1A and Table II. Plasmid pJO2 (p<sub>DPP1</sub>-lacZ) contains the DPP1 promoter fused to the lacZ gene of E. coli (17). Plasmid pGH210 replaces the EcoRI fragment of pJO2 with a truncated DPP1 promoter containing 0.44 kb of the upstream sequence (19). Using appropriate primers, a series of p<sub>DPP1</sub>-lacZ deletion plasmids were constructed for PCR amplification using plasmid pJO2 as the template. Each PCR product was digested with HindIII and EcoRI and substituted for the 0.87-kb HindIII/EcoRI fragment of pJO2. Plasmid constructions were confirmed by HindIII/EcoRI digestion.

Plasmid pJO2 contains a 4.3-kb GIS1 DNA fragment consisting of the native promoter (1.0 kb), the coding sequence (2.7 kb), and the 3′ untranslated region (0.6 kb). For this construction, 2.7- and 1.4-kb DNA fragments were amplified using genomic DNA as template with restriction sites incorporated at both 5′ and 3′ ends, respectively. The PCR products were digested with SacI/PstI or PstI/SacI as required. Plasmid YEp352 (28) was digested with SacI and SalI. The vector and both PCR fragments were purified by agarose gel electrophoresis and ligated at 16 °C for 15 h to generate plasmid pJO19. Plasmid construction was confirmed by restriction enzyme analysis. The GIS1 open reading frame (2.7 kb) was amplified by PCR using plasmid pJO19 as the template, and the PCR products were digested with XhoI and BamHI. This DNA fragment was cloned into the XhoI/BamHI site of...
plasmid pET-15b to generate plasmid pGHiS05. The correct in-frame fusion was confirmed by restriction enzyme analysis.

The Strategat QuikChange™ site-directed mutagenesis kit was utilized according to the manufacturer’s instructions to generate plasmids pJO20 through pJO26. These plasmids were derivatives of pJO2 (DPP1::His) and contained mutations in the three URS_PDS elements of the DPP1 promoter. The core of each site was mutated to the sequence 5′-AGGA-3′ (30). Each site was mutated to convert the sequence 5′-AGGA-3′ to 5′-AAAAA-3′. Mutagenesis was not performed directly with plasmid pJO2 because of the expense of the size limitations of this method. Accordingly, the DPP1 promoter of plasmid pJO2 was first subcloned into the EcoRI site of plasmid pPCR-Script AMP SK(+) to form plasmid pJO18. Single mutations in URS_PDS1, URS_PDS2, and URS_PDS3 were constructed using plasmid pJO18 as the template to make pJO18-S1, pJO18-S2, and pJO18-S3. All mutations were confirmed by DNA sequencing. The wild type DPP1 promoter in plasmid pJO2 was replaced with the mutated DPP1 promoters from pJO18-S1, pJO18-S2, and pJO18-S3 to form plasmids pJO20-pJO26. The orientation of the inserted fragments was verified with PstI digestion.

Construction of the gis1Δ and rph1Δ Mutants—The gis1Δ and rph1Δ mutants were constructed by the PCR-mediated gene deletion method of Brachmann et al. (31). Plasmids pRS413 and pRS415 were templates for PCR. The disruption DNA fragment for the GSI deletion (gis1Δ::LEU2) was amplified using pRS415 with primers containing the flanking sequences of the GSI gene. The disruption DNA fragment for the RPH1 deletion (rph1Δ::HIS3) was amplified using pRS413 with primers containing the flanking sequences of the RPH1 gene. These DNA fragments were transformed into W303-1A to replace the chromosomal copies of the GSI1 and RPH1 genes with the auxotrophic marker genes by homologous recombination (31). Deletion of each gene in the transformants was confirmed by PCR using appropriate primers for the GSI1 and RPH1 genes, respectively. The gis1Δ and rph1Δ mutants that we isolated were designated strains JOY37 and JOY38, respectively.

Expression and Purification of His-tagged Gis1p from E. coli—All steps in the purification of the recombinant Gis1p were performed at 4°C. E. coli cells containing the His-tagged Gis1p were washed once in 20 mM Tris-HCl (pH 8.0) buffer and suspended in 40 mM of 20 mM Tris-HCl (pH 8.0) buffer containing 0.5 mM NaCl, 5 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride. Cells were disrupted by a freeze-thaw cycle followed by two passes through a French press at 20,000 pounds/square inch. Unbroken cells and cell debris were removed by centrifugation at 12,000 × g for 40 min at 4°C. The cell extract (supernatant) was gently mixed with 2× with 2.5% slurry of Ni2+-NTA-agarose. The enzyme/Ni2+-NTA-agarose mixture was packed in a 10-mL PolyPrep disposable column. The column was washed with 10 mL of 20 mM Tris-HCl (pH 8.0) buffer containing 0.5 mM NaCl, 5 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride and then with 50 mL of 20 mM Tris-HCl (pH 8.0) buffer containing 0.5 mM NaCl, 250 mM imidazole, 10% glycerol, and 7 mM 2-mercaptoethanol. The His-tagged protein was then eluted from the column in 1-mL fractions with a total of 5 mL of 20 mM Tris-HCl (pH 8.0) buffer containing 0.5 mM NaCl, 250 mM imidazole, 10% glycerol, and 7 mM 2-mercaptoethanol.

Electrophoretic Mobility Shift Assays—Complementary single stranded oligonucleotides were annealed and used for electrophoretic mobility shift assays (Table III). The annealing reactions contained 10 μM oligonucleotides in a final volume of 0.1 mL. Samples were incubated for 2 min at 65°C and then for 15 min at 65, 37, 25, and 4°C. To facilitate labeling, the oligonucleotides were designed to leave a 5′-overhanging end after annealing. Approximately 1.5 μg of double stranded DNA was incubated with 5 units of Klenow fragment and [α-32P]dTMP (400–800 Ci/mmol) for 30 min at 30°C. Unincorporated dNTPs were separated from the labeled DNA using ProbeQuant G-50 Spin columns.

Electrophoretic mobility shift assays were performed as described previously (26). Briefly, electrophoretic mobility shift assays were performed in a 20-μL volume containing 4 pmol of radiolabeled DNA probe (1.75 × 108 cpm/pmol), 4 mM Tris-HCl (pH 8.0), 4 mM MgCl2, 1 mM dithiothreitol, 0.5 μg of poly(dI-dC), 6 μg of bovine serum albumin, 10% glycerol, and the indicated concentrations of recombinant Gis1p. DNA complexes to form at 30°C. The samples were mixed with 3 μl of loading solution (40% glucose, 0.25% bromphenol blue, 0.25% xylene cyanol) and resolved from free DNA by electrophoresis on 6% polyacrylamide gels (1.5-mm thickness) at 200 V for 2 h. Gels were dried onto blotting paper, and the reaction products were visualized by autoradiography.

Preparation of the Cell Extract and the Total Membrane Fraction—Yeast cells were suspended in 50 mM Tris-maleate buffer (pH 7.0) containing 1 mM Na2-EDTA, 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. Cells were disrupted by homogenization with chilled glass beads (0.5-mm diameter) using a Dounce homogenizer. Samples washed twice by centrifugation at 12,000 × g for 1 min, were sonicated for 1 min bursts followed by a 2-min cooling between bursts at 4°C. The cell extract was obtained by centrifugation of the homogenate at 1,500 × g for 10 min. The total membrane fraction was obtained by centrifugation of the cell extract at 100,000 × g for 2 h. Membranes were resuspended in buffer containing 50 mM Tris-maleate (pH 7.0), 10 mM MgCl2, 10 mM 2-mercaptoethanol, 20% glycerol (w/v), and 0.5 mM phenylmethylsulfonyl fluoride. Protein concentration of the cell extracts and membrane fractions were determined by the method of Bradford (32) using bovine serum albumin as the standard.

β-Galactosidase Assay—β-Galactosidase activity was assayed as described previously (33). The reaction mixture contained 100 mM sodium citrate buffer (pH 7.0), 1 mM ATP, 0.5 mM D-galactopyranoside, 1 mM MgCl2, 10 mM 2-mercaptoethanol, 20% glycerol (w/v), and 0.5 mM phenylmethylsulfonyl fluoride. Protein concentration of the cell extract and membrane fractions were determined by the method of Bradford (32) using bovine serum albumin as the standard.

DGPP Phosphatase Assay—β-32P DGPP was synthesized enzymatically with purified C. albicans tert-Butyl phosphatidate kinase (1) and used to raise antibodies in New Zealand White rabbits by standard procedures (34). The antibody was affinity purified using appropriate DGPP phosphatase using appropriate DGPP phosphatase activity was measured by following the release of water-soluble 32P from chloroform-soluble β-32P DGPP (12,500 cpm/nmol) as described by Wu et al. (1). The reaction mixture contained 50 mM citrate buffer (pH 5.0), 0.1 mM DGPP, 2 mM Triton X-100, and enzyme protein in a total volume of 0.1 mL. All enzyme assays were conducted at room temperature in triplicate. The average standard deviation of the assays was ± 5%. β-Galactosidase reactions were linear with time and protein concentration. A unit of β-galactosidase activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product per min.

Anti-DGPP Phosphatase Antibodies and Immunoblotting—The peptide sequence NVR5FIRKTFFNNIGARWR (residues 2–18 of the N-terminus of the DGPP phosphatase) was synthesized and used to raise antibodies in New Zealand White rabbits by standard procedures (34) at Bio-Synthesis, Inc. SDS-PAGE (35) using 12% slab gels and immunoblotting (36) with polyvinylidene difluoride membranes were performed as described previously. The blot was probed with a 1:500 dilution of the anti-DGPP phosphatase antibodies. Goat anti-rabbit IgG alkaline phosphatase conjugated secondary antibody at a dilution of 1:5000. The DGPP phosphatase protein was detected using the enhanced chemiluminescence Western blotting detection kit as described by the manufacturer. The DGPP phosphatase protein on immunoblots was acquired by FluorImaging analysis. The relative density of the protein was analyzed using ImageQuant software. The on the line range analysis.

Labeling and Analysis of Phospholipids—Labeling of phospholipids with 32P, was performed as described previously (37–39). Cells were grown to stationary phase in 5 mL of culture medium with 5 μCi/mL of 32P, Phospholipids were extracted from labeled cells by the method of

| Table III | DNA fragments used for electrophoretic mobility shift assays |
|-----------|-------------------------------------------------------------|
| Element   | **Annealed oligonucleotides**a                             |
| URS_PDS1  | 5′-GGACGTCTCTCTTAAGAGGCAACACCGGGCTT-3′                    |
| URS_PDS2  | 5′-GGAGCCGCGCTGATCCATTAAAAAGAGAAAAGTTTTTTCCAT-5′         |
| URS_PDS3  | 5′-TCTAGAAGCTTCAATAAAAAAACCAAGCCGTTT-3′                   |
| URS_PDS1 mutant | 5′-GGACGTCTCTCTTAAGAGGCAACACCGGGCTT-3′         |
| URS_PDS2 mutant | 5′-GGAGCCGCGCTGATCCATTAAAAAGAGAAAAGTTTTTTCCAT-5′         |
| URS_PDS3 mutant | 5′-TCTAGAAGCTTCAATAAAAAAACCAAGCCGTTT-3′                   |
Bligh and Dyer (40) and analyzed by two-dimensional thin layer chromatography. High performance silica gel plates were treated with 1% potassium oxalate dissolved in methanol/H2O (2:3) and baked at 100 ºC overnight to remove residual moisture. The solvent systems used in the first and second dimensions were chloroform/methanol/ammonium hydroxide/H2O (90:50:4:6) and chloroform/methanol/acetic acid/H2O (63:8:10:2), respectively. Plates were dried in vacuo for 2 h between the dimensions. The identity of the labeled phospholipids on the chromatography plates was confirmed by comparison with standard phospholipids after exposure to iodine vapor. Radiolabeled phospholipids were visualized by phosphorimaging analysis. The relative quantities of the phospholipids were analyzed using ImageQuant software.

Analyses of Data—Statistical analyses were performed with SigmaStat software. Statistical significance was determined by performing the Student’s t test. p values < 0.05 were taken as a significant difference.

RESULTS

Expression of P_\text{DPP1}-lacZ Reporter Genes with a Series of Promoter Deletions—Previous research in our laboratory (17) indicated that DGPP phosphatase activity increases in stationary phase cells and in cells supplemented with 50 µM inositol. This regulation has been ascribed to a transcriptional mechanism (17). To identify which regions of the DPP1 promoter were involved with this regulation, we constructed a series of P_\text{DPP1}-lacZ reporter genes with deletions from the 5’ end of the DPP1 promoter (Fig. 1A). Thus, analysis of changes in β-galactosidase activity resulting from deletions in the reporter genes could help identify promoter elements in DPP1 responsible for this regulation.

Wild type cells bearing the reporter genes were grown to the exponential and stationary phases in the absence and presence of 50 µM inositol, and cell extracts were assayed for β-galactosidase activity. As described previously (17), the expression of DPP1 was greater in stationary phase cells when compared with exponential phase cells (compare y-axis labels in Fig. 1, B and C). In exponential phase cells, DPP1 expression was induced by inositol supplementation until the promoter was deleted to −631 (Fig. 1B). Further deletion of the promoter to −441 showed comparable levels of DPP1 expression, but the inositol-mediated induction was abolished (Fig. 1B). However, deletion of the promoter to −394 resulted in a 4-fold increase in β-galactosidase activity (Fig. 1B), suggesting that a negative regulatory element was located between positions −441 and −394. Inspection of the promoter sequence revealed that this region contained a putative binding site for the transcription factor Gis1p. Deletion of the promoter to −292 resulted in a 2- to 2.5-fold decrease in the elevated levels of β-galactosidase activity (Fig. 1B). This was possibly because of the loss of an unidentified transcription activator site. In stationary phase cells, inositol supplementation resulted in a 4-fold increase in β-galactosidase activity when the promoter sequence was deleted to position −782 (Fig. 1C), which was not observed in exponential phase cells (Fig. 1B). This indicated the presence of another negative regulatory site between −809 and −782. This region also contains a putative binding site for Gis1p. For cells grown in the absence or presence of inositol, there was a 3.5- and 7.5-fold decrease in β-galactosidase activity, respectively, when the promoter sequence was deleted to position −492 (Fig. 1C). This was most likely because of the loss of the UAASZRE (41), which binds the transcription activator Zap1p to induce DPP1 expression (19). DPP1 expression increased 3.3- and 5.2-fold in stationary phase cells grown in the absence or presence of inositol, respectively, when the promoter sequence was deleted to position −394 (Fig. 1C). The induction of DPP1 expression in stationary phase (Fig. 1C) was consistent with the increase in expression observed in exponential phase (Fig. 1B) when the same region of the promoter was deleted. Under all growth conditions, β-galactosidase activity declined to low levels when the promoter sequence was deleted to −192 (Fig. 1, B and C).

Analysis of β-galactosidase activity from the P_\text{DPP1}-lacZ deletion reporter genes suggested that the loss of two Gis1p binding sites affected DPP1 expression, particularly in inositol-supplemented, stationary phase cells. The Gis1p binding site consensus (5’T(A/T)AGGGAT-3’) was identified previously (30) as an upstream activating sequence for post-diauxic shift

**FIG. 1.** Expression of P_\text{DPP1}-lacZ reporter genes with a series of promoter deletions in exponential and stationary phase cells grown in the absence and presence of inositol. Panel A, the indicated P_\text{DPP1}-lacZ reporter genes were constructed as described under “Experimental Procedures.” The Gis1p and Zap1p transcription factor binding sites in the DPP1 promoter are indicated. The full-length promoter construct contains 809 bases upstream of the ATG start site. The number of bases in each reporter gene construct is denoted at the left side of the figure. The names of the plasmids containing different sizes of the DPP1 promoter fused to lacZ are denoted at the right side of the figure. Panels B and C, wild type cells (strain W303–1A) bearing the indicated plasmids were grown in medium in the absence and presence of 50 µM inositol. Cells were harvested in exponential phase (1–2 × 10^8 cells/ml) (B) or stationary phase (1–2 × 10^9 cells/ml) (C). Cell extracts were prepared and assayed for β-galactosidase activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D.
we referred to the Gis1p binding site as URSPDS (for upstream repressing sequence during post-diauxic shift). In stationary phase cells supplemented with inositol, the \( \beta \)-galactosidase activity in gis1 mutant cells was 6.6-fold greater than that found in wild type cells (Fig. 2B).

Previous studies have noted the homology between Gis1p and another zinc finger transcription factor, Rph1p (23). Although the two proteins show only 35% similarity overall (23), the zinc finger regions that are thought to interact with DNA are 92.7% identical (23), suggesting that they bind similar DNA sequences. Gis1p and Rph1p have partially redundant roles in the repression of the photolyase gene PHR1 (23), and the consensus-binding site for Rph1p (5’-AGGGG-3’) is nearly identical to the core (5’-AGGGA-3’) of the Gis1p-binding URSPDS element (23). We examined the expression of DPP1 in rph1 mutant cells by measuring the \( \beta \)-galactosidase activity driven by the \( P_{ppp} \)-lacZ reporter gene. In exponential phase, rph1 mutant cells exhibited a small but significant (\( p < 0.05 \)) increase in DPP1 expression in the absence (1.3-fold) and presence (1.6-fold) of inositol (Fig. 2A). In stationary phase, rph1 mutant cells had 2.7-fold greater \( \beta \)-galactosidase activity in the absence of inositol and 2.6-fold greater activity in the presence of inositol when compared with control cells (Fig. 2B). From these data, we concluded that Rph1p also functions as a negative regulator of DPP1 expression. However, the regulatory effect of Gis1p was much greater than that of Rph1p on the regulation of DPP1. Accordingly, the role of Gis1p on the regulation of DPP1 was further studied.

**Effects of the gis1Δ Mutation and the Overexpression of the GIS1 Gene on the Expression of DGPP Phosphatase Activity**—We questioned whether the increase in DPP1 expression observed in gis1Δ mutant cells correlated with an increase in the levels of DGPP phosphatase activity. Because the gis1Δ-dependent changes in DPP1 expression were most evident in stationary phase cells grown with inositol, subsequent experiments were conducted under this growth condition. The DGPP phosphatase activity in the gis1Δ mutant was 2.5-fold greater when compared with the activity found in wild type cells (Fig. 3). We also questioned whether the overexpression of the GIS1 gene would have the opposite effect on the expression of DGPP phosphatase activity. Wild type and gis1Δ mutant cells were transformed with a multicopy plasmid bearing GIS1 (plasmid cultures grown without inositol, \( \beta \)-galactosidase activity was also 2-fold greater in gis1Δ mutant cells when compared with the control cells (Fig. 2B). The greatest effect on DPP1 expression occurred in stationary phase cells supplemented with inositol. The \( \beta \)-galactosidase activity in gis1Δ mutant cells was 6.6-fold greater than that found in wild type cells (Fig. 2B).

![Fig. 2](http://www.jbc.org/Downloadedfrom/fig2.png)

**Fig. 2.** Effects of the gis1Δ and rph1Δ mutations on the expression of the DPP1 gene in exponential and stationary phase cells grown in the absence and presence of inositol. Wild type (WT; strain W303–1A) gis1Δ mutant (strain JOY37), and rph1Δ mutant (strain JOY38) cells bearing the \( P_{ppp} \)-lacZ reporter gene (plasmid pJ02) were grown in medium in the absence and presence of 50 \( \mu \)M inositol. Cells were harvested in exponential phase (1–2 × 10⁶ cells/ml) (A) and stationary phase (1–2 × 10⁸ cells/ml) (B). Cell extracts were prepared and assayed for \( \beta \)-galactosidase activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D.
pJO19). The transformation efficiency with this plasmid was very low suggesting that overexpression of GIS1 may be toxic to cell growth. GIS1 toxicity has been observed previously (22) when the gene is overexpressed using an inducible promoter. The transformants that we did isolate may represent a population of cells where the copy number of the plasmid was reduced to a tolerable level. Nonetheless, the growth rate of these transformants was somewhat slower when compared with wild type or gis1Δ mutant cells. The DGPP phosphatase activity in gis1Δ mutant cells overexpressing GIS1 was 4.7- and 1.9-fold lower, respectively, when compared with the activity found in gis1Δ mutant cells and in wild type cells with the vector control (Fig. 3). Immunoblot analysis of membrane fractions using anti-DGPP phosphatase antibodies showed that the relative abundance of the DGPP phosphatase protein correlated with the levels of DGPP phosphatase activity (data not shown).

Effect of the gis1Δ Mutation on DPP1 Expression and DGPP Phosphatase Activity in Zinc-limited Cells—Previous studies have shown that the transcription factor Zap1p binds the DPP1 promoter and induces gene expression of exponential phase cells grown under zinc-limited conditions (19). We questioned whether Gis1p, a negative regulator of DPP1 expression, affected the expression of DPP1 under zinc-limited conditions in stationary phase cells. Wild type and gis1Δ mutant cells bearing the P<sub>DPP1</sub>-lacZ reporter gene were grown to the stationary phase in the presence and absence of zinc as described under “Experimental Procedures.” Cell extracts were assayed for β-galactosidase activity and DGPP phosphatase activity. Zinc limitation resulted in an increase in DPP1 expression (4.7-fold) and DGPP phosphatase activity (3.8-fold) in wild type cells (Fig. 4). The extent of induction of DPP1 (4.7-fold) and DGPP phosphatase activity (3.7-fold) by zinc limitation in gis1Δ mutant cells was essentially identical to that observed in wild type cells (Fig. 4). However, the total levels of DPP1 expression and DGPP phosphatase activity in gis1Δ mutant cells were 3.2- and 2.3-fold greater, respectively, when compared with wild type cells (Fig. 4). These results indicated that the gis1Δ mutation did not affect the Zap1p-mediated induction of DPP1 expression in zinc-limited cells.

Gis1p Binds to the URS<sub>PDG</sub> Sites in the DPP1 Promoter in Vitro—We sought evidence that Gis1p mediates the regulation of DPP1 expression by direct interaction with the DPP1 promoter. This interaction was examined by electrophoretic mobility shift assays using His-tagged Gis1p and oligonucleotides containing each of the three URS<sub>PDG</sub> elements in the DPP1 promoter. The labeled oligonucleotide probes containing each of the three URS<sub>PDG</sub> elements migrated to a position near the top of the polyacrylamide gel in the presence of His-Gis1p (Fig. 5A). This result indicated that Gis1p formed a complex with the oligonucleotide probes. Furthermore, formation of these complexes was dependent on the concentration of Gis1p (Fig. 5A). Another Gis1p-DNA complex migrated to a position just above the unbound DNA probe (Fig. 5A). This result from complexes formed with a truncated portion of Gis1p. SDS-PAGE analysis of the purified recombinant Gis1p indicated that partial proteolysis of Gis1p occurred during purification. Electrophoretic mobility shift assays were also performed with Gis1p and DNA probes where the core of each URS<sub>PDG</sub> element containing the sequence 5′-AGGGA-3′ was changed to 5′-AAAAA-3′. The formation of Gis1p-DNA complexes was abolished when the three binding sites were mutated (Fig. 5B). These results demonstrated that the URS<sub>PDG</sub> in the DPP1 promoter was required for Gis1p binding in vitro.

Effects of URS<sub>PDG</sub> Element Mutations on DPP1 Expression—The effects of mutations in the three URS<sub>PDG</sub> elements on DPP1 expression were examined in vivo. Full-length P<sub>DPP1</sub>-lacZ reporter genes were constructed with individual mutations in the three URS<sub>PDG</sub> elements where the core sequence of 5′-AGGGA-3′ was changed to 5′-AAAAA-3′. Wild type cells bearing the wild type or mutant P<sub>DPP1</sub>-lacZ reporter genes were grown to stationary phase in the presence of 50 μM inositol, and cell extracts were assayed for β-galactosidase activity. DPP1 promoters containing mutations in the URS<sub>PDG</sub><sup>1</sup>, URS<sub>PDG</sub><sup>2</sup>, and URS<sub>PDG</sub><sup>3</sup> elements yielded in an increase in β-galactosidase activity of 3.3-, 3.3-, and 3.7-fold, respectively, when compared with the wild type DPP1 promoter (Fig. 6). These results indicated that any one of the URS<sub>PDG</sub> elements played a role in regulating DPP1 expression in vivo. The effect of the three URS<sub>PDG</sub> mutations made in combination on the expression of DPP1 was not significantly different from that observed for any one of the individual mutations (data not shown).

Effect of the gis1Δ Mutation on Phospholipid Composition—We questioned whether the regulation of expression of DGPP phosphatase activity by Gis1p affected the level of DGPP in vivo. Accordingly, the effect of the gis1Δ mutation on the phospholipid composition of stationary phase cells grown in the presence of 50 μM inositol was examined. Cells were labeled to steady state with [32P]<sub>γ</sub> and phospholipids were extracted and analyzed by two-dimensional thin-layer chromatography. As described previously (1, 17), DGPP accounted for 0.3% of the major phospholipids in wild type cells (Fig. 7, inset). The amount of DGPP decreased by 78% in gis1Δ mutant cells (Fig. 7, inset). This corresponded to the increased DGPP phospha-
tase activity observed in gis1Δ mutant cells (Fig. 3). The gis1Δ mutation also affected the levels of the major phospholipids phosphatidylethanolamine and phosphatidylcholine. When compared with wild type cells, gis1Δ mutant cells exhibited a 24% increase in the amount of phosphatidylethanolamine and a 39% decrease in phosphatidylcholine (Fig. 7). The amounts of the other phospholipids in the gis1Δ mutant were not significantly different from that of wild type cells.

DISCUSSION

DGPP phosphatase activity is present in a wide range of organisms, including bacteria, yeast, plants, and mammals (42). The conservation of this enzymatic activity in such highly divergent species suggests that DGPP phosphatase may play an important role in cell physiology. The DPP1-encoded (9) DGPP phosphatase in S. cerevisiae has been purified to homo-

geneity and characterized with respect to its enzymological and kinetic properties (1). DGPP phosphatase catalyzes the removal of the β-phosphate from DGPP to yield phosphatidate and then removes the phosphate from phosphatidate to generate diacylglycerol (1). The enzyme also exhibits a phosphatidate phosphatase activity in the absence of DGPP (1). Studies with dpp1Δ mutant cells have shown that DGPP phosphatase plays a role in regulating phospholipid metabolism (16, 17). Mutant cells exhibit elevated levels of phosphatidate and DGPP and reduced levels of phosphatidylinositol. In addition, the substrates and products of DGPP phosphatase (DGPP, phosphatidate, and diacylglycerol) have lipid messenger roles in higher eukaryotic cells (7, 10, 43). Thus, DGPP phosphatase may play a role in lipid signaling by regulating specific pools of these molecules (1, 10).

Examination of DGPP phosphatase expression indicates that DPP1 is induced by growth phase, inositol supplementation, and zinc starvation (17, 19). The induction of DPP1 in zinc-
limited cells is mediated by the Zap1p transcription factor, which controls expression through its binding to a UASZAP gene in the DPP1 promoter (19). To identify promoter elements responsible for regulation of DPP1 by growth phase and inositol supplementation, we analyzed the β-galactosidase activity expressed in cells bearing a series of P_dpp1-lacZ deletion reporter genes. Unfortunately, this analysis did not allow us to identify promoter elements that control DPP1 induction in response to growth phase and inositol supplementation. Although exponential phase DPP1 expression no longer responded to inositol supplementation with promoter sequences that were 0.63 kb or less, the same reporter genes showed inositol induction in stationary phase cells. In addition, all reporter genes consistently showed higher DPP1 expression in stationary phase. The promoter elements regulating DPP1 induction by growth phase and inositol supplementation would not have been identified if they were within the first 192 bases upstream of the start codon. Moreover, the reporter gene product, a non-native protein in yeast, may not be subjected to degradation or turnover in the same manner as mRNA.

Analysis of the P_dpp1-lacZ deletion reporter genes resulted in the identification of Gin1p as a negative regulator of DPP1 expression. Deletion of two Gin1p binding sites (URS1PDeG and URSPD3) within the DPP1 promoter resulted in an increase in DPP1 expression, whereas URSPD3 was identified by inspection of the DPP1 promoter sequence. Studies with the gis1Δ mutant were performed to address the hypothesis that Gin1p was the transcription factor involved in regulating DPP1 expression under all conditions examined; the greatest increase (6.6-fold) occurred in inositol-supplemented stationary phase cells. The increase in DPP1 expression was accompanied by an increase in expression of DGPP phosphatase activity. Electrophoretic mobility shift assays using purified recombinant Gin1p verified that the URSPD3 sequences in the DPP1 were Gin1p binding sites. Moreover, mutations in the three URSPD3 elements within the DPP1 promoter abolished Gin1p-DNA interactions in vitro and abolished the regulation of DPP1 in vivo.

Gin1p (44) is a positive regulator of the heat shock protein genes SSA3, HSP12, and HSP26 (22, 30) and a negative regulator of the photolyase PHR1 gene (23). The expression of Gin1p is relatively low during the exponential phase of growth and maximally expressed as cells progress into stationary phase (22). Thus, it was not surprising that the effect of the gis1Δ mutation on DPP1 expression was most evident after the diauxic shift. In addition, the strongest effect of the gis1Δ mutation was observed in inositol-supplemented media. Perhaps GIS1 expression was greatest in cells grown with inositol. However, additional studies will be necessary to address this hypothesis.

Because Gin1p regulated DGPP phosphatase activity, we examined the effect of the gis1Δ mutation on phospholipid composition. Consistent with the elevated expression of DGPP phosphatase activity, the gis1Δ mutant exhibited a 76% reduction in the level of DGPP when compared with that of wild type cells. It was not possible to verify a corresponding increase in the level of phosphatidate (product of the DGPP phosphatase reaction), because the change in the level of DGPP was within the standard deviation of phosphatidate measurements. The gis1Δ mutant also exhibited alterations in the amounts of the major phospholipids phosphatidylethanolamine and phosphatidylcholine. In gis1Δ mutant cells, there was a 39% decrease in the level of phosphatidylethanolamine and a 24% increase in the level of phosphatidylcholine when compared with wild type cells. The mechanism responsible for these changes was unclear. Changes in phosphatidylethanolamine and phosphatidylcholine composition may be a consequence of a signaling function of DGPP brought about by the regulation of DGPP phosphatase expression. Alternatively, Gin1p may regulate the expression of other genes involved in phospholipid metabolism. For example, activation of phospholipid methyltransferase activities, which convert phosphatidylethanolamine to phosphatidylcholine (45, 46), in gis1Δ mutant cells could account for increasing phosphatidylcholine at the expense of phosphatidylethanolamine. Future studies will address these questions.

In summary, the work reported in this communication supported the conclusion that the DPP1-encoded DGPP phosphatase of S. cerevisiae was negatively regulated by the transcription factor Gin1p. Regulation of DPP1 expression by Gin1p was most evident in stationary phase cells grown in the presence of inositol. These growth conditions (17), and the condition of zinc limitation (19), are stressful states whereby DGPP phosphatase is induced. We hypothesize that Gin1p may function to attenuate the induction of DGPP phosphatase to maintain some steady-state level of DGPP within the cell.

REFERENCES
1. Wu, W.-I., Liu, Y., Riedel, B., Wissing, J. B., Fischl, A. S., and Carman, G. M. (1996) J. Biol. Chem. 271, 1866–1876
2. Wissing, J. B., and Behrbeh, H. (1993) FEBS Lett. 315, 85–95
3. Munnik, T., Meijer, H. J. G., Riet, B., Hur, H., Frank, W., Bartels, D., and Musgrave, A. (2000) Plant J. 22, 147–154
4. Munnik, T., de Vrieze, T., Irvine, R. F., and Musgrave, A. (1996) J. Biol. Chem. 271, 15708–15715
5. Den Hartog, M., Musgrave, A., and Munnik, T. (2001) Plant J. 25, 55–65
6. Van der Luit, A. H., Piatti, T., Van Doorn, A., Musgrave, A., Felix, G., Boller, T., and Munnik, T. (2000) Plant Physiol. 123, 1507–1515
7. Balboa, M., Balsinde, J., Dillon, D. A., Carman, G. M., and Dennis, K. A. (1999) J. Biol. Chem. 274, 522–526
8. Dillon, D. A., Chen, X., Zeitz, G. M., Wu, W.-I., Waggerman, D. W., Dewald, J., Brindley, D. N., and Carman, G. M. (1997) J. Biol. Chem. 272, 10361–10366
9. Toke, D. A., Bennett, W. L., Dillon, D. A., Chen, X., Oshiro, J., Ostrander, D. B., Wu, W.-I., Creasman, A., Voelker, D. B., Fischl, A. S., and Carman, G. M. (1998) J. Biol. Chem. 273, 3278–3284
10. Brindley, D. N., and Waggerman, D. W. (1998) J. Biol. Chem. 273, 24281–24284
11. Brindley, D. N., English, D., Plügl, C., Buri, K., and Lang, Z. C. (2002) Biochim. Biophys. Acta 1582, 33–44
12. Stukey, J., and Carman, G. M. (1997) Protein Sci. 6, 469–472
13. Toke, D. A., McClintick, M. L., and Carman, G. M. (1999) Biochemistry 38, 14696–14713
14. Dillon, D. A., Wu, W.-I., Riedel, B., Wissing, J. B., Dowhan, W., and Carman, G. M. (1996) J. Biol. Chem. 271, 30548–30553
15. Pauliukner, A. J., Chen, X., Hargraves, D. J., Schurr, K., Waeche, C. J., Carman, G. M., and Sternweis, P. C. (1999) J. Biol. Chem. 274, 14831–14837
16. Toke, D. A., Bennett, W. L., Oshiro, J., Wu, W.-I., Voelker, D. B., and Carman, G. M. (1999) J. Biol. Chem. 274, 14331–14335
17. Oshiro, J., Jangsaengwony, S., Chen, X., Han, G.-S., Quinn, J. E., and Carman, G. M. (2000) J. Biol. Chem. 275, 40887–40896
18. Werner-Washburne, M., Braun, E., Johnson, G. C., and Singer, R. A. (1993) Microbiol. Rev. 57, 383–401
19. Han, G.-S., Johnston, C. N., Chen, X., Athenstaedt, K., Daum, G., and Carman, G. M. (2001) J. Biol. Chem. 276, 10126–10133
20. Lyonse, T. J., Gess, A. P., Gaither, L. A., Beaton, D. P., and Eide, D. J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7907–7902
21. Yuan, D. S. (2000) Genetics 156, 45–58
22. Pedrazzi, J., Burckert, N., Egger, P., and De Virgilio, C. (2000) EMBO J. 19, 2569–2579
23. Jung, Y. K., Wang, L., and Sancar, A. G. (1999) Mol. Cell. Biol. 19, 7630–7638
24. Rose, M. D., Winston, F., and Heitz, P. (1990) Methods in Yeast Genetics: A Laboratory Course Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
25. Culbertson, M. R., and Henry, S. A. (1975) J. Biol. Chem. 250, 5791–5800
26. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989, 2nd Ed., Cold Spring Harbor, NY, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. coli, 14, 58–60
28. Craven, G. R., Steers, J. E., and Anfinsen, C. B. (1965) J. Biol. Chem. 240, 2468–2477
29. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring
35. Laemmli, U. K. (1970) *Nature* **227**, 680–685
36. Haid, A., and Suissa, M. (1983) *Methods Enzymol.* **96**, 192–205
37. Atkinson, K., Fogel, S., and Henry, S. A. (1980) *J. Biol. Chem.* **255**, 6653–6661
38. Atkinson, K. D., Jensen, B., Kolat, A. I., Storm, E. M., Henry, S. A., and Fogel, S. (1980) *J. Bacteriol.* **141**, 558–564
39. McDonough, V. M., Buxeda, R. J., Bruno, M. E. C., Ozier-Kalogeropoulos, O., Adeline, M.-T., McMaster, C. R., Bell, R. M., and Carman, G. M. (1995) *J. Biol. Chem.* **270**, 18774–18780
40. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
41. Zhao, H., Butler, E., Rodgers, J., Spizzo, T., Duesterhoeft, S., and Eide, D. (1998) *J. Biol. Chem.* **273**, 28713–28720
42. Carman, G. M. (1997) *Biochim. Biophys. Acta* **1348**, 45–55
43. Hansum, Y. A., Loomis, C. R., and Bell, R. M. (1985) *J. Biol. Chem.* **260**, 10039–10043
44. Balciunas, D., and Ronne, H. (1999) *Mol. Gen. Genet.* **262**, 589–599
45. Carman, G. M., and Henry, S. A. (1999) *Prog. Lipid Res.* **38**, 361–399
46. Kent, C., and Carman, G. M. (1999) *Trends Biochem. Sci.* **24**, 146–150
47. Thomas, B., and Rothstein, R. (1989) *Cell* **56**, 619–630
48. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122**, 19–27
