Regulation of the *Saccharomyces cerevisiae* DPP1-encoded Diacylglycerol Pyrophosphate Phosphatase by Zinc*

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The DPP1 gene, encoding diacylglycerol pyrophosphate (DGPP) phosphatase from *Saccharomyces cerevisiae*, has recently been identified as a zinc-regulated gene, and it contains a putative zinc-responsive element (UASZRE) in its promoter. In this work we examined the hypothesis that expression of DGPP phosphatase was regulated by zinc availability. The deprivation of zinc from the growth medium resulted in a time- and dose-dependent induction of β-galactosidase activity driven by a P$_{DPP1}$-lacZ reporter gene. This regulation was dependent on the UASZRE in the *DPP1* promoter and was mediated by the Zap1p transcriptional activator. Induction of the DGPP phosphatase protein and activity by zinc deprivation was demonstrated by immunoblot analysis and measurement of the dephosphorylation of DGPP. The regulation pattern of DGPP phosphatase in mutants defective in plasma membrane (Zrt1p and Zrt2p) and vacuolar membrane (Zrt3p) zinc transporters indicated that enzyme expression was sensitive to the cytoplasmic levels of zinc. DGPP phosphatase activity was inhibited by zinc by a mechanism that involved formation of DGPP-zinc complexes. Studies with well-characterized subcellular fractions and by indirect immunofluorescence microscopy revealed that the DGPP phosphatase enzyme was localized to the vacuolar membrane.

The *DPP1*-encoded diacylglycerol pyrophosphate (DGPP)$^1$ phosphatase (1) is a membrane-associated enzyme from the yeast *Saccharomyces cerevisiae* that catalyzes the removal of the β-phosphate from DGPP to form PA and then removes the phosphate from PA to form DG (2). DGPP is a minor phospholipid in *S. cerevisiae* (2) that contains a pyrophosphate group attached to DG (3). DGPP is derived from PA via the reaction catalyzed by PA kinase (2, 4). DGPP is postulated to function in a novel lipid-signaling pathway (5, 6). Metabolic labeling studies with plants, where DGPP was first discovered (3), show that DGPP accumulates upon G protein activation (7) and upon hyperosmotic stress (8, 9). Exogenous DGPP augments secretion of prostaglandins in mouse macrophages (10). This occurs by a mechanism that involves the activation of cytosolic phospholipase A$_2$ via the mitogen-activated protein kinase pathway, an important event in the immunoinflammatory response of leukocytes (10). The accumulation of DGPP in plants is short-lived (9); it is rapidly converted to PA and then to DG (11), consistent with the reactions catalyzed by yeast DGPP phosphatase (2).

*DPP1*-encoded DGPP phosphatase also exhibits a PA phosphatase activity (2), but DGPP is the preferred substrate, with a specificity constant 10-fold higher than that of PA (2). The DGPP phosphatase protein (1) contains a three-domain phosphatase sequence motif (12–14) that is conserved in a superfamily of lipid phosphatase enzymes (15–19). Conserved Arg$^{125}$, His$^{169}$, and His$^{223}$ residues within domains 1, 2, and 3, respectively, play important roles in the phosphatase reactions catalyzed by the enzyme (20). The DGPP phosphatase enzyme also utilizes lysophosphatidate (15), sphingolipid phosphates (21), and isoprenoid phosphates (22) as substrates *in vitro*. However, only DGPP and PA have been shown to be substrates *in vivo* (1).

We have begun to examine the regulation of DGPP phosphatase expression in *S. cerevisiae* to gain an understanding of DGPP function. The enzyme is induced by inositol in both exponential and stationary phase cells (23). DGPP phosphatase expression is greater in stationary phase cells, and the inositol-and growth phase-dependent regulation of the enzyme is additive (23). These growth conditions have profound effects on the expression of many phospholipid biosynthetic enzymes and on the regulation of phosphatidylinositol metabolism (5, 24–26). Interestingly, DGPP phosphatase regulation by inositol occurs in a manner that is opposite to that of many phospholipid biosynthetic enzymes (23). Studies performed with a *dpp1Δ* mutant reveal that DGPP phosphatase plays a role in the regulation of phospholipid metabolism by inositol as well as regulating the cellular levels of DGPP, PA, and phosphatidylinositol (16, 23). Moreover, inhibition of DGPP phosphatase activity by CDP-diacylglycerol and stimulation of phosphatidylserine synthase activity by DGPP may contribute to this regulation on a biochemical level (23).

In this work, we showed that DGPP phosphatase expression was also regulated by zinc availability. Zinc deprivation induced DGPP phosphatase in wild-type cells. This regulation was mediated by the Zap1p transcriptional activator through a zinc-responsive element (UASZRE) in the promoter of the *DPP1* gene. The pattern of DGPP phosphatase regulation in mutants defective in plasma membrane and vacuolar membrane zinc...
transporters indicated that enzyme expression was sensitive to the cytoplasmic levels of zinc. DGPP phosphatase activity was inhibited by zinc through a mechanism that involved formation of DGPP-zinc complexes. We also showed that DGPP phosphatase was localized to the vacuolar membrane.

**Experimental Procedures**

**Materials**—All chemicals were reagent grade. Growth medium supplies were purchased from Difco Laboratories. Yeast nitrogen base lacking zinc sulfate was purchased from BIO 101 by custom order. Triton X-100, bovine serum albumin, benzamidine, phenylmethylsulfonyl fluoride, aprotinin, leupeptin, pepstatin, O-nitrophenyl β-d-galactopyranoside, 4-(2-pyridylazo)resorcinol, and poly-L-lysine were purchased from Sigma. Restriction endonucleases, modifying enzymes, and Vent DNA polymerase were obtained from New England Biolabs. Oligonucleotides for PCR and DNA sequencing were prepared by Genosys Biotechnologies, Inc. Protein assay reagents, electrophoresis reagents, DNA and nucleotides for PCR and DNA sequencing were prepared by Bio-Rad. DNA and protein molecular mass markers were from Life Technologies and Bio-Rad, respectively. Polyvinylidene difluoride membranes, protein A-Septarose, and the enhanced chemiluminescence Western blotting detection kit were purchased from Amersham Pharmacia Biotech. Mouse monoclonal anti-HA antibodies (12CA5) and ImmunoPure fluorochrome conjugated goat anti-mouse IgG (H+L) antibodies were purchased from Pierce, respectively.

**Glass beads and unbroken cells** were removed by centrifugation at 25,000 × g for 10 min. The supernatant (cell extract) was used for enzyme assays and immunoblot analysis.

**DNA Manipulations, Amplification of DNA by PCR, Site-directed Mutagenesis, and DNA Sequencing**—Plasmid DNA preparation, restriction enzyme digestion, and DNA ligations were performed by standard methods (28). Transformation of yeast (29) and E. coli (28) were performed as described previously. Conditions for the amplification of DNA by PCR were optimized as described previously (30).

**Site-directed mutagenesis** was performed by PCR using overlap extension (31) or using the QuikChange site-directed mutagenesis kit. DNA sequencing reactions were performed by the dioxyde method using Taq polymerase (28).

**Plasmid Constructions**—The plasmids used in this study are listed in Tables I and II. Plasmid pJH201, which contains the DPP1 promoter fused to the lacZ gene of E. coli, was constructed as described previously (23).

**Enzyme Assays**—DGPP phosphatase activity was measured by following the release of water-soluble 32P, from chloroform-soluble [32P]DGPP (10,000–15,000 cpm/nmol) as described by Wu et al. (2). Protein concentration was determined by the method of Bradford (32) using bovine serum albumin as the standard.

**Plasmid pHG209** was constructed by replacing the EcoRI fragment of pJH201 with the DPP1 promoter containing mutations in the putative UASZRE and its upstream sequence, which was generated by PCR using overlap extension. A 0.39-kb 5′-fragment of the DPP1 promoter containing the mutation was amplified by PCR (primers: 5′-GTGAAAGGACGAGATTCTATAAAGGGGACACCGG-3′ and 5′-TAAACCGATGTAATCTATGCTGAAAAGGTGTG-3′). The two PCR products were mixed, denatured, annealed, and extended. The overlap-extended DNA fragment was then amplified by PCR (primers: 5′-GTGAAAGGACGAGATTCTATAAAGGGGACACCGG-3′ and 5′-GTTTTTTAATTACGTTACGTTGCATCTTTTGTCG-3′). The 0.85-kb PCR products of the DPP1 promoter containing EcoRI and substituted for the EcoRI fragment of pJH201. Plasmid pHG210 was constructed by replacing the EcoRI fragment of pJH201 with the DPP1 promoter lacking the UASZRE and its upstream sequence, which was generated by PCR (primers: 5′-CCCGGATATGGTTGACGTTCCGCATTCTTTA-3′ and 5′-GTTTTTTAATTACGTTACGTTGCATCTTTTGTCG-3′). Plasmids pHG201 and pHG202 contain the DPP1 gene with sequences for a HA epitope tag inserted after the start codon. A 0.69-kb DNA fragment containing the DPP1 promoter, the start codon, and an HA epitope sequence was amplified with the primers 5′-CTCTAGAGTCGACCCAGTTTGGTCG-3′ and 5′-AGCGTAGTCTGGGACGTCGTATG-3′. The two PCR products were mixed, denatured, annealed, and extended. The overlap-extended DNA fragment was then amplified using primers 5′-CTCTAGAGTCGACCCAGTTTGGTCG-3′ and 5′-GTTTTTTAATTACGTTACGTTGCATCTTTTGTCG-3′. The 2-kb PCR products of the HA-DPP1 DNA were digested with SalI and inserted into the same restriction site in plasmids pSB415 and YEp51, generating pHG201 and pHG202, respectively. All plasmid constructions were confirmed by DNA sequencing.

**Preparation of Cell Extracts, DGPP Phosphatase, and Protein Determination**—Cells were disrupted with glass beads (32) in 50 mM Tris-maleate buffer (pH 7.0) containing 1 mM NaEDTA, 0.3 M 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 mixed with 2-mercaptoethanol, 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 (34). 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. DGPP phosphatase and β-galactosidase assays were conducted at 30 and 25 °C, respectively. The average S.D. of the enzyme volume of 0.1 ml. DGPP phosphatase and complexes with zinc, such as Na₂EDTA, compete with 4-(2-pyridyl)anilines (38) to resorcinol for zinc and cause a decrease in absorbance.

Immunoblot Analysis—SDS-polyacrylamide gel electrophoresis (35) using either 10 or 12% slab gels and immunoblotting (36) using polyvinylidene difluoride membranes were performed as described previously. The molecular mass standards used were phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Rabbit polyclonal anti-DGPP phosphatase antibodies (37) and mouse monoclonal anti-HA antibodies were used at a dilution of 1:1000. Anti-rabbit and mouse IgG-alkaline phosphatase conjugate were used as secondary antibodies at a dilution of 1:5000. DGPP phosphatase protein was detected on immunoblots 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. Immunoblot signals were in the linear range of detectability.

Analysis of DGPP-Zinc Complexes—The ability of DGPP to form complexes with zinc ions was analyzed with the metal indicator reagent 4-(2-pyridyl)azo)resorcinol by a modification of a method described by Hunt et al. (38). Reaction mixtures contained 40 mM HEPES buffer (pH 7.0), 50 μM 4-(2-pyridyl)azo)resorcinol, 100 μM ZnCl₂, and various concentrations of diactanoyl DGPP. In this assay, the reaction of 4-(2-pyridyl)azo)resorcinol with zinc ions produced a red color that was measured spectrophotometrically at 500 nm. Compounds that form complexes with zinc, such as NαEDTA, compete with 4-(2-pyridylazo)resorcinol for zinc and cause a decrease in absorbance.

Isolation and Characterization of Subcellular Fractions—Organelles were isolated from wild-type cells grown in YEPD growth medium to early stationary phase at 30 °C under aerobic conditions. Mitochondria, microsomes 1 (30,000 × g fraction), 2 (40,000 × g fraction), 3 (100,000 × g fraction), cytosol, vacuoles, and lipid particles were isolated from spheroplasts, which were prepared by standard methods (39). The mitochondrial and microsomal fractions were isolated from cell extracts by differential centrifugation (40). The cytosol was the supernatant of the 100,000 × g fraction (40). Vacuoles and lipid particles were isolated by several steps of flotation and density gradient centrifugation as described by Uchida et al. (41) with the modifications of Leber et al. (42). Plasma membranes were prepared from cells disrupted with glass beads using a combination of differential centrifugation and density gradient centrifugation (43). Relative enrichment and cross-contamination of organelle fractions were assessed by immunoblot analysis (36) as described by Zinner and Daum (44). Antibodies were directed against porin (mitochondria), Sec61p (microsomes, endoplasmic reticulum), carbonic anhydrase (cytosol), cathepsin D (vacuoles), Erg6p (lipid particles), plasma membrane ATPase (plasma membrane), and glyceraldehyde 3-phosphate dehydrogenase (cytosol).

Immunofluorescence Microscopy—Immunofluorescent staining of cells was performed as described by Pringle et al. (45) with minor modifications. Wild-type strain W303-1A, carrying either plasmid pGH202 or plasmid pGH202, was grown to the late exponential phase of growth in SC medium and then fixed for 1 h with 3.7% formaldehyde. Fixed cells were treated with zymolase (1 unit/ml) for 15 min and then attached to a polylysine-coated glass slide. Cells were treated with Triton X-100 for 10 min followed by incubation for 20 min with phosphate-buffered saline (pH 7.5) containing 5% bovine serum albumin. The cells on the glass slide were incubated for 1 h with anti-HA antibodies (15 μg/ml), washed with phosphate-buffered saline, and then incubated for 1 h with fluorescein-conjugated anti-IgG (H + L) antibodies (7.5 μg/ml). Images were observed and recorded using an Olympus BH2-RFC1 fluorescence microscope equipped with a Photometrics Sensys KAF-1400 CCD camera.

RESULTS

Effect of Zinc Deprivation on the Expression of β-Galactosidase Activity in Cells Bearing the P_DGPP-lacZ Reporter Gene—Yuan (46) recently identified the DPP1 gene in a genetic screen designed to identify genes that are regulated by zinc deprivation. In addition, inspection of the DPP1 gene revealed that it contains a sequence (ACCTGAAAAGT) in its promoter (−442 to −452) that is closely related to a consensus UAS ZRE (47). The UAS ZRE is a cis-acting element that affects the transcriptional activation of several genes (48), including ZRT1, ZRT2, and ZRT3, in response to zinc deprivation (47). These observations were the basis for the hypothesis that DGPP phosphatase was regulated by zinc availability. We examined the expression of DPP1 in zinc-limited growth media. This analysis was facilitated by the use of plasmid pJO2 that bears a P_DGPP-lacZ reporter gene where the DPP1 promoter is fused in-frame with the coding sequence of the E. coli lacZ gene (23). The expression of β-galactosidase activity in cells with plasmid pJO2 is dependent on transcription driven by the DPP1 promoter (23). Wild-type cells bearing plasmid pJO2 were grown to the exponential phase of growth in SC (zinc replete) medium. The cells were washed, resuspended in SC medium without zinc, and incubated for 12 h. Control cells were resuspended in SC medium. The removal of zinc from the growth medium resulted in a time-dependent increase in β-galactosidase activity (Fig. 1). At the time of maximum expression, the β-galactosidase activity from cells grown in the absence of zinc was 3-fold greater than the activity from the control cells grown in the presence of zinc (Fig. 1). Over the time course of this experiment, there was no difference in the growth rate of the two cultures (data not shown).

To further examine the effect of zinc on the expression of the DPP1 gene, we measured β-galactosidase activity from wild-type cells bearing plasmid pJO2 that were grown with various concentrations of zinc. In these experiments and those described below, cells were first grown for 24 h in zinc-deplete media to reduce the intracellular levels of zinc. This regimen described below, cells were first grown for 24 h in zinc-deplete media to reduce the intracellular levels of zinc. This regimen was used to accentuate the regulation of the DPP1 gene by zinc deprivation. Zinc was then added to the final concentrations indicated. Reduction for zinc in the growth medium resulted in a dose-dependent increase in β-galactosidase activity. The activity found in cells grown in the absence of zinc was 64-fold greater that the activity in cells grown in the presence of 5 μM zinc (Fig. 2).

The specific activity of β-galactosidase in cells grown in the presence of 1.5 μM zinc, the approximate concentration of zinc in SC medium, was 0.09 units/mg. There was a 1.8-fold reduction in β-galactosidase activity when the concentration of zinc

| Plasmid | Relevant characteristics | Reference |
|---------|--------------------------|-----------|
| pJO2    | P_DGPP-lacZ in YEp357R   | 23        |
| pGH209  | pJO2 with mutations in the UAS ZRE | This study |
| pGH210  | pJO2 without the UAS ZRE  | This study |
| pGH201  | HA-tagged DPP1 gene ligated into the SaI site of pRS415 | This study |
| pGT2-DPP1 | HA-tagged DPP1 gene ligated into the SaI site of Yepl51 | This study |
| pDGNC10 | DPP1 gene ligated into the SaI site of Yepl51 | 47        |
| pGNC50  | DPP1 gene ligated into the SaI site of Yepl51 | 47        |
| pRS415  | DPP1 gene ligated into the SaI site of Yepl51 | 47        |
| YEp351  | DPP1 gene ligated into the SaI site of Yepl51 | 47        |
| YEp351  | DPP1 gene ligated into the SaI site of Yepl51 | 47        |
| YEp351  | DPP1 gene ligated into the SaI site of Yepl51 | 47        |

TABLE II

Plasmids used in this work

| Plasmid | Relevant characteristics | Reference |
|---------|--------------------------|-----------|
| pJO2    | P_DGPP-lacZ in YEp357R   | 23        |
| pGH209  | pJO2 with mutations in the UAS ZRE | This study |
| pGH210  | pJO2 without the UAS ZRE  | This study |
| pGH201  | HA-tagged DPP1 gene ligated into the SaI site of pRS415 | This study |
| pGT2-DPP1 | HA-tagged DPP1 gene ligated into the SaI site of Yepl51 | This study |
| pDGNC10 | DPP1 gene ligated into the SaI site of Yepl51 | 47        |
| pGNC50  | DPP1 gene ligated into the SaI site of Yepl51 | 47        |
| pRS415  | DPP1 gene ligated into the SaI site of Yepl51 | 47        |
| YEp351  | DPP1 gene ligated into the SaI site of Yepl51 | 47        |
| YEp351  | DPP1 gene ligated into the SaI site of Yepl51 | 47        |
| YEp351  | DPP1 gene ligated into the SaI site of Yepl51 | 47        |
| YEp351  | DPP1 gene ligated into the SaI site of Yepl51 | 47        |
was increased to 5 μM (Fig. 2). Concentrations of zinc up to 1 mM did not cause a further reduction in β-galactosidase activity (data not shown).

Effect of the Putative UAS ZRE on the Expression of β-Galactosidase Activity in Cells Bearing the PDPP1-lacZ Reporter Gene in Response to Zinc Deprivation—The effect of mutations in the putative UAS ZRE in the DPP1 promoter on the regulation by zinc was examined. The putative UAS ZRE in the PDPP1-lacZ reporter plasmid pJO2 was changed to a nonconsensus sequence by transition and transversion mutations (plasmid pGH209) and was deleted from the DPP1 promoter (plasmid pGH210). Cells bearing these reporter plasmids were precultured in zinc-deplete media and then incubated in media without and with 1.5 μM zinc. These mutations did not affect the expression of β-galactosidase activity when cells were grown in the absence of zinc (Fig. 3). In contrast to the control, cells bearing the mutations in the UAS ZRE and lacking the UAS ZRE did not exhibit the great induction of β-galactosidase activity when grown in the absence of zinc (Fig. 3).

Regulation of DPP1 by Zinc Deprivation in a Mutant Defective in the ZAP1 Regulatory Gene—Zap1p is a transcriptional activator that is maximally expressed in zinc-deplete cells and repressed in zinc-replete cells (49). Zap1p binds to the UAS ZRE of the ZRT1, ZRT2, and ZRT3 genes for increased expression in zinc-deplete media (47, 49, 50). We examined whether the regulation of DPP1 by zinc was dependent on Zap1p function. A zap1Δ mutant bearing plasmid pJO2 was grown in the absence and presence of 1.5 μM zinc followed by the measurement of β-galactosidase activity. In contrast to wild-type cells, the absence of zinc in the growth medium did not result in the induction of β-galactosidase activity (Fig. 4). As a control, we examined the expression of β-galactosidase activity in wild-type and zap1Δ mutant cells bearing the reporter plasmid pDG2. This plasmid contains one UAS ZRE from the ZRT1 gene (47). As described previously (47), β-galactosidase activity was induced in wild-type cells but was not induced in zap1Δ mutant cells when they were grown in the absence of zinc (Fig. 4).

Effect of Zinc Deprivation on the Levels of DGPP Phosphatase Activity and Protein—We examined the regulation of expression of the DPP1-encoded DGPP phosphatase by zinc. Wild-type cells were first precultured in zinc-deplete media followed by incubation in growth media containing zinc. The depletion of zinc from the growth media led to a dose-dependent induction of DGPP phosphatase activity (Fig. 5A). DGPP phosphatase specific activity in cells grown in the absence of zinc was 10-fold greater than the activity from cells grown with 5 μM zinc (Fig. 5A).

We also examined the levels of the DGPP phosphatase protein in cells grown in the absence and presence of zinc. For these experiments, we used an epitope-tagged DPP1 allele in the single-copy plasmid pGH201. In this plasmid, the sequence for HA was inserted into the N terminus of the DPP1-coding sequence. Plasmid pGH201 was expressed in a dpp1A mutant to avoid interference from the DGPP phosphatase encoded by the genomic wild-type copy of the DPP1 gene (1). This plasmid directed normal levels of DGPP phosphatase activity (data not shown). The epitope-tagged DGPP phosphatase protein was specifically recognized by anti-HA antibodies at the expected molecular mass of about 34 kDa. Immunoblot analysis showed that the levels of the DGPP phosphatase protein increased 10-fold in a dose-dependent manner when zinc was depleted from the growth medium (Fig. 5B).

Regulation of DGPP Phosphatase Activity and Protein by Zinc in Mutants Defective in Genes Encoding Zinc Transporters—The regulation of DGPP phosphatase was examined in mutants defective in the ZRT1, ZRT2, ZRT3, COT1, and ZRC1 genes. Zrt1p (51) and Zrt2p (52) are high affinity and low affinity plasma membrane zinc transporters, respectively. Zrt3p (50) is a vacuolar membrane zinc efflux transporter, whereas Cot1p and Zrc1p are vacuolar membrane zinc influx transporters (50, 53). For cells grown in the presence of zinc, expression of DGPP phosphatase activity was 4.3- and 2-fold greater in a zrt1Δ zrt2Δ double mutant and in a zrt3Δ mutant, respectively, when compared with the wild-type control (Fig. 6A). Expression of DGPP phosphatase activity in a cot1Δ zrc1Δ double mutant grown in the presence of zinc was not significantly different from that in wild-type cells (Fig. 6A). Deprivation of zinc from the growth medium of the mutants resulted in a further increase in DGPP phosphatase activity that was comparable with the level of activity found in wild-type cells grown without zinc (Fig. 6A). Expression of the DGPP phosphatase protein was also examined in the zinc transporter mutants. For these experiments, the enzyme protein was analyzed by immunoblotting using anti-DGPP phosphatase antibodies. The pattern of regulation for DGPP phosphatase protein expression (Fig. 6B) in the mutants was consistent with the activity found in these cells (Fig. 6A).
Effect of Zinc on DGPP Phosphatase Activity and Formation of DGPP-Zinc Complexes—We examined the effect of zinc ions on DGPP phosphatase activity. The addition of zinc to the assay system resulted in a dose-dependent inhibition of DGPP phosphatase activity (Fig. 7). The zinc-mediated inhibition of activity followed cooperative kinetics (Fig. 7, inset). Analysis of the data according to the Hill equation yielded an IC50 value for zinc of 150 \( \mu M \) and a Hill number of 2.5. Owing to the fact that DGPP contains a pyrophosphate group, we questioned whether the mechanism of inhibition involved formation of DGPP-zinc complexes. Formation of DGPP-zinc complexes was examined using the metal indicator reagent 4-(2-pyridylazo)resorcinol. For these experiments, we used dioctanoyl DGPP because of its solubility in aqueous solutions. Dioctanoyl DGPP is enzymatically active when employed as a substrate for pure DGPP phosphatase (10). The addition of DGPP to a 50 \( \mu M \) 4-(2-pyridylazo)resorcinol, 100 \( \mu M \) ZnCl2 mixture resulted in a dose-dependent decrease in absorbance at 500 nm, indicating the formation of DGPP-zinc complexes (data not shown). The concentration of DGPP that resulted in half-maximum complex formation was 90 \( \mu M \). This concentration was within the range of the IC50 value for zinc. Formation of Na2EDTA-zinc complexes was used as a control. Under the same conditions, the concentration of Na2EDTA that resulted in half-maximum Na2EDTA-zinc complex formation was 30 \( \mu M \).

Subcellular Localization of DGPP Phosphatase—DGPP phosphatase has been purified from crude microsomal membranes (2). However, the subcellular location of DGPP phosphatase has not been addressed. To better understand the functional role(s) of DGPP phosphatase, we examined the subcellular localization of the enzyme. Subcellular fractions of S. cerevisiae were isolated and characterized as described under “Experimental Procedures.” The fractions were then used for immunoblot analysis using anti-DGPP phosphatase antibodies. The 34-kDa DGPP phosphatase protein (Dpp1p) was highly enriched in isolated vacuoles (Fig. 8). The subcellular location of DGPP phosphatase was examined further using wild-type cells that
representative of two independent experiments. The immunoblot data shown is that was found with wild-type strain CM100 was the same as that protein in wild-type cells grown in the presence of zinc was set at 1. Data by scanning densitometry. The amount of the DGPP phosphatase protein (Dpp1p) bands on the immunoblot was quantified using M zinc sulfate. Cultures were then diluted to 1 \times 10^6 cells/ml in zinc-deplete media and grown to the exponential phase of growth (A_{600 nm} \sim 0.5) in the absence and presence of 5 \mu M zinc sulfate. Cell extracts were prepared and used for the assay of DGPP phosphatase activity (panel A) and subjected to immunoblot analysis using a 1:1000 dilution of anti-DGPP phosphatase antibodies (panel B). Each activity data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments \pm S.D. The density of the DGPP phosphatase protein (Dpp1p) bands on the immunoblot was quantified by scanning densitometry. The amount of the DGPP phosphatase protein in wild-type cells grown in the presence of zinc was set at 1. Data that was found with wild-type strain CM100 was the same as that found with wild-type strain DY1457. The immunoblot data shown is representative of two independent experiments.

FIG. 6. Effect of zrt1\Delta zrt2\Delta, zrt3\Delta, and cot1\Delta zrc1\Delta mutations on the regulation of DGPP phosphatase activity and protein by zinc deprivation. Wild-type (WT; strain DY1457) cells, zrt1\Delta zrt2\Delta (strain ZHY3), zrt3\Delta (strain CM101), and cot1\Delta zrc1\Delta (strain CM104) mutant cells were grown for 24 h in SC growth media containing 5 \mu M zinc sulfate. Cultures were then diluted to 1 \times 10^6 cells/ml in zinc-deplete media and grown to the exponential phase of growth (A_{600 nm} \sim 0.5) in the absence and presence of 5 \mu M zinc sulfate. Cell extracts were prepared and used for the assay of DGPP phosphatase activity (panel A) and subjected to immunoblot analysis using a 1:1000 dilution of anti-DGPP phosphatase antibodies (panel B). Each activity data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments \pm S.D. The density of the DGPP phosphatase protein (Dpp1p) bands on the immunoblot was quantified by scanning densitometry. The amount of the DGPP phosphatase protein in wild-type cells grown in the presence of zinc was set at 1. Data that was found with wild-type strain CM100 was the same as that found with wild-type strain DY1457. The immunoblot data shown is representative of two independent experiments.

FIG. 7. Effect of zinc on DGPP phosphatase activity. DGPP phosphatase activity was measured using pure enzyme with the indicated concentrations of ZnCl_2. The inset is a replot of the zinc-mediated inhibition of DGPP phosphatase activity.

expressed the HA-tagged version of the enzyme directed by the single-copy and multicopy plasmids pGH201 and pGH202, respectively. Cells were grown to exponential phase, fixed, and probed for the HA epitope by indirect immunofluorescence microscopy. The fluorescence signal in cells with the single-copy plasmid was difficult to detect when compared with the signal in cells with the multicopy plasmid. HA-DGPP phosphatase fluorescence in cells with the multicopy plasmid outlined the periphery of the vacuole (Fig. 9B), which could be observed by phase contrast microscopy (Fig. 9A). In separate experiments, identification of the vacuole was confirmed by red fluorescence resulting from the styryl dye FM 4-64, which specifically stains the vacuolar membrane (54). These data provided evidence that the DGPP phosphatase enzyme was localized to the vacuolar membrane.

DISCUSSION

The DPP1 gene was first identified by Toke et al. (1) as the gene encoding DGPP phosphatase. This identification was based on amino acid sequence information derived from the purified enzyme (1). Recent studies identify DPP1 as a gene that is regulated by zinc deprivation (46, 48). The work presented here advances the understanding of the regulation of DGPP phosphatase by zinc and the localization of the enzyme. Using a P_DPP1-lacZ reporter gene, we showed that the expression of DPP1 was induced in a time- and dose-dependent manner when wild-type cells were deprived of zinc. The DPP1 promoter contains a putative UAS_{ZRE} (47), which is present in several genes that are regulated by zinc deprivation (48). The regulation of DPP1 expression by zinc was dependent on this UAS_{ZRE}. Mutations in the element, as well as the deletion of the element from the P_DPP1-lacZ reporter gene, precluded the regulation by zinc deprivation. The DPP1 gene is also induced when cells are supplemented with inositol and when they enter the stationary phase of growth. This regulation is not dependent on the UAS_{ZRE},^2 Induction of UAS_{ZRE}-containing genes such as ZRT1, ZRT2, and ZRT3, in response to zinc deprivation (47) is mediated by the Zap1p transcriptional activator (49). Indeed, Zap1p mediated DPP1 regulation by zinc deprivation. As previously reported (46, 48), the regulation of DPP1 by zinc deprivation was lost in a zap1\Delta mutant background.

We examined the regulation of DGPP phosphatase expression in response to zinc directly by measuring the levels of DGPP phosphatase activity and protein. The depletion of zinc from the growth medium of wild-type cells resulted in a 10-fold increase in DGPP phosphatase activity, which corresponded to a 10-fold increase in the level of the DGPP phosphatase protein. The magnitude of induction of the DGPP phosphatase enzyme was considerably less than the 64-fold induction of \beta-galactosidase activity driven by the P_DPP1-lacZ reporter gene. However, the regulation of the DGPP phosphatase enzyme was more consistent with that of DPP1 mRNA abundance (46, 48).

^2 J. Oshiro and G. M. Carman, unpublished data.
Zinc homeostasis in *S. cerevisiae* is largely controlled by Zrt1p and Zrt2p, which are plasma membrane proteins that transport extracellular zinc into the cytoplasm (51, 52). Expression of Zrt1p and Zrt2p is induced when the extracellular concentration of zinc is high (51, 52). Moreover, when the zinc concentration is low, the Zrt1p transporter is a stable plasma membrane protein (55). However, when the zinc concentration is high, Zrt1p is ubiquitinated (56) and removed from the plasma membrane by endocytosis and vacuolar degradation (55). Cytoplasmic levels of zinc are controlled further by the vacuolar membrane efflux (Zrt3p) and influx (Cop1p and Zrc1p) zinc transporters (50). Expression of DGPP phosphatase activity in the *zrt1Δ zrt2Δ* mutant grown in the presence of zinc was significantly greater (4.3-fold) than that found in wild-type cells. The *zrt3Δ* mutation in the vacuolar membrane zinc efflux transporter also caused an increase (2-fold) in expression of DGPP phosphatase activity, but to a smaller magnitude. These data were consistent with the hypothesis that expression of DGPP phosphatase was sensitive to the cytoplasmic levels of zinc.

**DGPP1-encoded DGPP phosphatase activity is independent of any divalent cation requirement (2).** However, DGPP phosphatase activity is inhibited by manganese, calcium, and magnesium ions (2). In this work, we showed that the enzyme was inhibited by zinc ions. The inhibitor constant for zinc ions (150 μM) was within its cellular concentration (150–1500 μM, based on a cell volume of 5 × 10⁻¹³ liter/cell), determined for cells grown with varying concentrations of zinc (47, 55). Thus, regulation of DGPP phosphatase activity by zinc may be physiologically relevant. Pyrophosphate compounds have the ability to chelate divalent cations (57). Our studies indicated that DGPP had the ability to form complexes with zinc. This observation was consistent with a mechanism of inhibition of DGPP phosphatase that involved the formation of DGPP-zinc complexes.

Based on studies using well characterized subcellular fractions and indirect immunofluorescence microscopy, the DGPP phosphatase enzyme was localized to the vacuolar membrane. DGPP phosphatase is predicted to be an integral membrane protein with six transmembrane-spanning regions distributed over its entire protein sequence (1). The three domains that comprise the active site of DGPP phosphatase (20) are positioned on the same side of the membrane (1). The internal pH of the vacuole and the pH optimum for DGPP phosphatase activity are acidic, suggesting that the active site of the enzyme may be located on the internal surface of the vacuolar membrane. Additional studies will be required to address this hypothesis and establish the topology of the enzyme within the membrane.

Zinc is an essential mineral (58). It is a cofactor for more than 300 enzymes, including superoxide dismutase, carbonic anhydrase, and alcohol dehydrogenase, as well as several proteases (58). In addition, several proteins, including transcription factors, require zinc as a structural cofactor for folding of specific domains (59). Notwithstanding its essential nature, zinc can be toxic if it is accumulated in excess amounts (58). The cytoplasmic levels of zinc are controlled by a variety of mechanisms, including cellular influx (60), efflux (61, 62), and chelation by metallothioneins (63). As discussed above, the plasma membrane zinc transporters play a major role in this regulation. Why is a vacuolar membrane-associated DGPP phosphatase activity regulated in a coordinate manner with zinc transporters whose main function is to regulate zinc homeostasis? The **DGPP1** gene is not essential (1). Moreover, **dpp1Δ** mutants do not exhibit any dramatic phenotypes under a variety of growth conditions (1), including fluctuations in zinc supplementation (data not shown) (46). Thus, the role of DGPP phosphatase in response to zinc deprivation would have to be complimentary to other mechanisms that respond to this stress. DGPP phosphatase plays a role in regulating the levels of DGPP, PA, and phophatidylinositol in *S. cerevisiae* (16, 23). Although the function of DGPP is unclear, it is tempting to speculate that it may function to chelate a specific pool of zinc ions at the surface of the vacuolar membrane. This function would be eliminated by the dephosphorylation of free DGPP by the phosphatase, especially under zinc-limiting conditions. An alternative role for the enzyme may be to control the levels of PA and phosphatidylinositol in vacuolar membranes. These two phospholipids play critical roles in the structure and function of membranes in *S. cerevisiae* and in higher eukaryotic cells (5, 24, 25). Accordingly, future studies will address the role DGPP phosphatase plays in the regulation of vacuolar phospholipid metabolism in response to zinc deprivation.

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