Vacuole Membrane Topography of the DPP1-encoded Diacylglycerol Pyrophosphate Phosphatase Catalytic Site from Saccharomyces cerevisiae

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The Saccharomyces cerevisiae DPP1-encoded diacylglycerol pyrophosphate phosphatase is a vacuole membrane-associated enzyme that catalyzes the removal of the β-phosphate from diacylglycerol pyrophosphate to form phosphatidate, and it then removes the phosphate from phosphatidate to form diacylglycerol. The enzyme has six putative transmembrane domains and a hydrophilic region that contains a phosphatase motif required for its catalytic activity. In this work, we examined the topography of diacylglycerol-pyrophosphate phosphatase catalytic site within the transverse plane of the vacuole membrane. Results of protease protection analysis using endoproteinase Lys-C and labeling of cysteine residues using sulphydryl reagents were consistent with a model where the catalytic site of diacylglycerol-pyrophosphate phosphatase was oriented to the cytosolic face of the vacuole membrane. In addition, diacylglycerol-pyrophosphate phosphatase activity was found with intact vacuoles. The phospholipids diacylglycerol pyrophosphate (0.6 mol %) and phosphatidate (1.4 mol %) were found in the vacuole membrane, and their levels decreased to an undetectable level and by 79%, respectively, when cells were depleted for zinc. The reduced levels of diacylglycerol pyrophosphate and phosphatidate correlated with the induced expression of diacylglycerol-pyrophosphate phosphatase. This work suggested that diacylglycerol pyrophosphate phosphatase functions to regulate the levels of diacylglycerol pyrophosphate and phosphatidate on the cytosolic face of the vacuole membrane.

DGPP1 phosphatase, encoded by the DPP1 gene (1–3), is a 34-kDa vacuole membrane-associated enzyme (4) that was first discovered in the yeast Saccharomyces cerevisiae (5). It catalyzes the removal of the β-phosphate from DGPP to form PA, and it then removes the phosphate from PA to form diacylglycerol (Fig. 1) (5). DGPP is the preferred substrate, and PA will not serve as a substrate in the presence of DGPP (5). DGPP phosphatase also utilizes lyso-PA (6), sphingoid base phosphates (7), and isoprenoid phosphates (2) as substrates in vitro. However, only DGPP and PA have been shown to be substrates in vivo (8). In fact, the products of the LCB3/LBP1/YSR2 (9–11) and the CWI8 (12) genes are responsible for the dephosphorylation of sphingoid base phosphates and isoprenoid phosphates in vivo, respectively.

The DGPP molecule was originally identified in plants as the product of the PA kinase enzyme (13). Research with plants indicates that DGPP may function as a signaling molecule under stress conditions. It accumulates upon G-protein activation (14), hyperosmotic stress (15), dehydration (15), Rhizobium-secreted nodulation factors (16), and general elicitors such as xylanase (17). DGPP accumulation is transient and coincides with a rise in PA levels (15, 17). Because of the reactions catalyzed by DGPP phosphatase, the enzyme may play a role during stress conditions to regulate specific cellular pools of DGPP and PA (18). Indeed, the Arabidopsis thaliana homolog (AtLPP1) of the yeast DPP1 gene is induced by genotoxic stress (γ and UV-B radiation), G-protein activation, and oxidative stress (19). Consistent with these observations, the S. cerevisiae DPP1-encoded DGPP phosphatase is induced by the stress conditions of zinc depletion (4) and stationary phase (20) and by inositol supplementation (20). Moreover, the expression of the DPP1 homolog in the pathogenic yeast Candida albicans is induced in clinical isolates that are resistant to azole antifungal agents (21).

The catalytic activity of DGPP phosphatase is conferred by a three-domain lipid phosphatase motif (22) found in the lipid phosphatase superfamily (23, 24). The conserved Arg (25), His (26), and His residues within domains I, II, and III, respectively, are required for both the DGPP phosphatase and PA phosphatase activities of the enzyme (25). The lipid phosphatase activities of DGPP phosphatase are Mg2+-dependent and N-ethylmaleimide-insensitive (5). The PA phosphatase activity of the DGPP phosphatase is distinct (5) from the Mg2+-dependent and N-ethylmaleimide-sensitive PA phosphatase enzyme (26, 27) that is presumably responsible for the synthesis of phospholipids and triacylglycerols in S. cerevisiae (28). In addition to yeast, DGPP phosphatase activity is found in a wide range of organisms (e.g. plants, bacteria, and mammalian cells (19, 29)), suggesting an important role for the enzyme in cell physiology (28).

Deletion of the DPP1 gene results in altered phospholipid composition (8). dpp1Δ mutant cells exhibit increased amounts of DGPP and PA and a decrease in the amount of PI (8). Moreover, DPP1 is one of the most highly regulated genes that respond to zinc depletion in the S. cerevisiae genome (3, 30). However, the physiological role of the DGPP phosphatase enzyme is not fully understood because dpp1Δ mutant cells do not exhibit dramatic phenotypic changes under standard laboratory conditions (1–3). The subcellular location of DGPP phos-
phatase to the vacuole membrane suggests that the functional role of the enzyme utilizing phospholipid substrates should be governed by the topography of its catalytic site. In this report, we provided evidence based on protease protection analysis, chemical labeling, and enzyme activity measurements that the DGPP phosphatase catalytic site was oriented to the cytosolic face of the vacuole membrane. In addition, we demonstrated that the enzyme substrates DGPP and PA were present in the vacule membrane and that their levels were significantly reduced when cells were depleted for zinc.

**EXPERIMENTAL PROCEDURES**

**Materials—**All of the chemicals were reagent grade. Growth medium included 2% glucose. The appropriate amino acid of synthetic complete medium supplemented with 1.4 μM zinc sulfate. Cultures were harvested, washed in de-ionized distilled water, diluted to 1 x 10^6 cells/ml in media containing 0 or 1.4 μM zinc sulfate, and grown for 24 h. Cultures were then diluted to 1 x 10^6 cells/ml and grown in media containing 0 or 1.4 μM zinc sulfate. This process was used to deplete internal stores of zinc. For metabolic labeling experiments, yeast was washed with Liqui-Nox and rinsed several times with distilled water followed by a final rinse with de-ionized distilled water.

**Preparation of Cell Extracts and Vacuoles—**Cell extracts were prepared as described previously (34, 35). Cells were suspended in 50 mM Tris maleate buffer (pH 7.0) containing 1 mM EDTA, 0.3 mM sucrose, 10 mM 2-mercaptoethanol, 0.01% benzamidine, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin. Cells were disrupted by homogenization with chilled glass beads (0.5-mm diameter) using a Biospec Products bead beater. Samples were homogenized for 10-min bursts followed by a 2-min cooling between bursts at 4°C. The cell extract (supernatant) was obtained by centrifugation of the homogenate at 15,000 x g for 10 min. Vacuoles were isolated from spheroplasts by several steps of flotation and density gradient centrifugation as described by Uchida et al. (36). The protein concentration of the cell extract and vacuoles was determined by the method of Bradford (37) using bovine serum albumin as the standard.

**Staining of Vacuoles with FM4-64—**Vacuolar membranes were stained with the specific styryl dye FM4-64 (38) at a final concentration of 50 μM. Fluorescent images were observed and recorded using an Olympus BH2-RFCA fluorescence microscope equipped with a Photometrics Sensys KAF-1400 CCD camera.

**SDS-PAGE and Immunoblot Analysis—**Proteins were separated by SDS-PAGE (39) using 10% gels or 10% SDS-PAGE with a 10% gradient gel. Membranes were probed with an appropriate dilution of the indicated primary antibody. Goat anti-rabbit or anti-mouse IgG alkaline phosphatase conjugate was used as a secondary antibody against primary antibodies produced in rabbits or mice, respectively, at a dilution of 1:5000. Antibody interactions were detected using the enhanced chemiluminescence West Pico detection kit as described by the manufacturer. Signals on the membrane were acquired by fluoromaging analysis. The relative density of the protein signals was analyzed using ImageQuant software. Immunoblot signals were in the linear range of detectability.

**Endoprotease Lys-C Treatment of Vacuoles—**Vacuoles (5 μg of protein) were mixed with 1 μg of endoprotease Lys-C and incubated for 2 h at 37°C in a total volume of 15 μl. The reaction mixture contained 50 mM Tris-HCl (pH 8.5), 0.3% sucrose, and 3% urea. Urea was included in the reaction mixture, because it facilitated exposure of lysine residues to endoprotease Lys-C and provided some protection from the endogenous vacuolar protease(s) that digested the DGPP phosphatase-depleted vacuole. Vacuoles were nonspecifically labeled by including 0.4% Triton X-100 in the reaction mixture. After incubation, 5 mM phenylmethylsulfonyl fluoride was added to the mixture to inhibit the protease. The reaction products were then subjected to SDS-PAGE with a 10–20% gradient gel followed by transfer to polyvinylidene difluoride membrane. The membrane was probed with anti-HA antibodies or with antibodies raised against the C-terminal epitope (residues 263–279) of DGPP phosphatase (20).

**Chemical Labeling of Cysteine Residues—**The cysteine residues of vacuole membrane proteins were labeled with sulphydryl reagents as described by Leng et al. (41) with some modifications. Vacuoles (15 μg of protein) were suspended in 200 μl of Tris-HCl buffer (pH 7.4) containing 0.5% sucrose and incubated for 10 min at 25°C. To ensure absence or presence of 100 μM AMS, a membrane-impermeable reagent used to label cysteine residues, the reaction mixture was then diluted to 1 ml with vacuole suspension buffer and incubated for 20 min at 25°C with 250 μM AMS, a membrane-permeable reagent used to label cysteine residues with Bolton-Hunter reagent (7). The labeling reaction was terminated by the addition of Tris-HCl buffer (pH 7.4) to a final concentration of 50 mM Tris-HCl. The labeled vacuoles were collected by centrifugation at 37,000 x g for 30 min at 4°C. The vacuoles were washed once with 500 μl of radioimmunoprecipitation buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) (42) and mixed with 100 μl of 10% slurry of agarose-conjugated anti-HA antibodies for 2 h at 4°C. The antibody-antigen affinity matrix was washed by centrifugation at 5,000 x g for 30 s, washed twice with radioimmunoprecipitation buffer, and resuspended in 20 μl of SDS-PAGE treatment buffer. After boiling and a brief centrifugation, the supernatant was subjected to SDS-PAGE with a 10–20% gradient gel followed by transfer to polyvinylidene difluoride
membrane. The membrane was probed with alkaline phosphatase-conjugated NeutAvidin to detect biotinylated proteins. The membrane was then stripped and re-probed with anti-HA antibodies to detect DGPP phosphatase.

Preparation of Labeled DGPP Substrates—32P-Labeled DGPP was enzymatically synthesized from PA and ATP with purified Catharanthus roseus PA kinase as described by Wu et al. (5). [32P]DGPP was synthesized from PA and [γ-32P]ATP, whereas [α-32P]DGPP was synthesized from [32P]PAP and ATP. The [32P]PAP used in the latter reaction was synthesized from diacglycerol and [γ-32P]ATP using Escherichia coli diacylglycerol kinase (43). The 32P-labeled DGPP substrates were purified by thin-layer chromatography on potassium oxalate-treated plates using the solvent system chloroform/acetone/methanol/glacial acetic acid/water (50:15:13:12:4) (5).

DGPP Phosphatase Assays—DGPP phosphatase activity was routinely measured by following the release of water-soluble 32P, from chloroform-soluble [β-32P]DGPP (10,000 cpm/nmol) (5). Unless otherwise indicated, the standard reaction mixture contained 50 mM citrate buffer (pH 5.0), 0.1 mM [32P]DGPP, 2 mM Triton X-100, and enzyme protein in a total volume of 0.1 ml. The function of Triton X-100 in the assay system is to solubilize the DGPP substrate (5). All of the enzyme assays 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.

DGPP phosphatase activity was also measured with DGPP incorporated into phospholipid vesicles that were prepared by sonication (44). The phospholipid vesicles contained 0.18 mM PC, 0.125 mM PE, 0.125 mM PI, 0.062 mM PS, and 0.1 mM [α-32P]DGPP. The molar ratio of PC/PE/PS in the vesicles was 3:2:2:1, the approximate composition of the major phospholipids in wild type S. cerevisiae (45). The molar ratio of the PC/PE/PS mixture to DGPP was 5:1. In this assay, DGPP phosphatase activity was measured by following the formation of [32P]PI from [32P]DGPP (10,000 cpm/nmol) (5). Following the reaction, phospholipid vesicles (supernatant) were separated from vacuoles by centrifugation at 37,000 × g for 30 min. Phospholipids were extracted (46) and analyzed by thin-layer chromatography using chloroform/acetone/methanol/glacial acetic acid/water (50:15:13:12:4) as the solvent system (5). The identity of the labeled phospholipids on the chromatography plates was confirmed by comparison with standard radioactive DGPP and PA. Radiolabeled DGPP and PA were visualized by phosphorimaging analysis, and their relative quantities were analyzed using ImageQuant software.

Analysis of Vacuole Membrane Phospholipids—Phospholipids were extracted from vacuoles by the method of Bligh and Dyer (46). The aqueous phase and mat of solid material between the two phases of this extraction were removed with a Pasteur pipette and subjected to a second extraction with chloroform. The chloroform phases were combined, dried under nitrogen, and resuspended in 500 μl of chloroform/methanol (9:1, v/v). A 25-μl aliquot was used to determine the total phosphatide content of the sample. The remainder was dried to a minimal volume under nitrogen. The sample was spotted onto an oxalate-EDTA (1.2% potassium oxalate, 2 mM EDTA dissolved in methanol/water (2:3, v/v)) treated high performance TLC Silica Gel 60 plate, which had been heated at 110 °C overnight and cooled to room temperature immediately prior to the application of the sample. The first dimension was developed using chloroform/methanol/ammonium hydroxide/water (30:16:7:1.3:2, v/v). The plate was placed in the tray of the chamber that was flooded with anhydrous ammonia (47). Plates were sprayed with sulfuric acid/methanol (1:1, v/v) and heated at 120 °C for 20 min. This treatment caused the phospholipids to become purple-gray in color while the rest of the plate remained white. Vacuole phospholipids were identified by comparison to standard phospholipid Silica gel containing phospholipids was scraped from the plate and collected directly into glass-ignition tubes. Distilled, de-ionized water (300 μl) and 70% perchloric acid (150 μl) were added directly to the silica gel. The samples were heated at 150 °C overnight to digest the phospholipids. After cooling to room temperature, the digested sample was filtered with an Acrodisc 4-mm syringe filter and subjected to phosphate determination as described by Ames and Dubin (48). Potassium phosphate was used as the standard.

Analyses of Data—Statistical analyses were performed with SigmaPlot software. Statistical significance was determined by performing the Student’s t test. p values < 0.05 were taken as a significant difference.
Fig. 3. Enrichment of the HA-tagged DPP1-encoded DGPP phosphatase in purified vacuoles. dpp1Δ mutant cells with and without the DPP1HA allele on a multicopy plasmid were grown to exponential phase. Cells were harvested, and vacuoles were isolated as described under “Experimental Procedures.” Panel A, 5 μg of cell extract (E) and vacuoles (V) were subjected to SDS-PAGE using a 10% slab gel followed by transfer to polyvinylidene difluoride membrane. The membrane was probed with anti-HA antibodies to detect the HA-tagged DGPP phosphatase (Dpp1pHA) and antibodies against the 100-kDa subunit of the vacuole H^+-ATPase (Vph1p) and the 61-kDa carboxypeptidase Y (Prc1p). The data are representative of three independent vacuole preparations. Panel B, DGPP phosphatase activity was measured with the indicated fractions under standard assay conditions using [γ-32P]DGPP as substrate. Each data point represents the average of triplicate determinations ± S.D.

cell extract (Fig. 3A). Faster migrating forms of the DGPP phosphatase protein in the vacuoles were recognized by the anti-HA antibodies (Fig. 3A). This indicated that the enzyme was subject to proteolytic cleavage at its C-terminal end during the purification of vacuoles. The presence of protease inhibitors during the vacuole preparation did not prevent this proteolysis.

The enrichment of DGPP phosphatase activity in the purified vacuoles was examined. Cell extracts and vacuoles from the dpp1Δ mutant expressing the HA-tagged enzyme were assayed for DGPP phosphatase activity under standard assay conditions. The specific activity of DGPP phosphatase in vacuoles (2.6 units/mg) was 16.8-fold greater than the activity (0.154 units/mg) in the cell extract (Fig. 3B). The LPP1 gene of S. cerevisiae encodes a Golgi membrane-associated (50) lipid phosphatase enzyme that also exhibits DGPP phosphatase activity (8). To examine the possible contribution of the LPP1-encoded enzyme to the DGPP phosphatase activity in vacuoles, we measured activity in vacuoles purified from dpp1Δ mutant cells that did not bear the DPP1HA allele. The vacuoles derived from the dpp1Δ mutant did not possess any measurable DGPP phosphatase activity (Fig. 3B).

DGPP Phosphatase Activity of Intact and Ruptured Vacuoles—Activity measurements of a membrane-associated phospholipid synthetic enzyme before and after organelle disruption with detergent have been used to determine the topography of the enzyme catalytic site (51, 52). The lack of significant enzyme activity with an intact organelle preparation and a significant amount of latent activity following organelle rupture implies that the catalytic site faces the lumen of that organelle (51, 52). Likewise, the expression of a significant amount of activity with the intact organelle coupled to a lack of latent activity following rupture implies that the catalytic site faces the external face of the organelle (51, 52). To address whether the DGPP phosphatase catalytic site was oriented to the cytosolic or the luminal surface of the vacuole membrane, enzyme activity was measured using intact and ruptured vacuoles. Throughout this work, vacuole integrity was confirmed by microscopic examination of vacuoles stained with the fluorescent dye FM4–64. Intact vacuoles exhibited a fluorescent signal that was concentrated at the periphery of the vacuoles (Fig. 4). Upon rupture, the fluorescent signal of the dye dissipated. A significant amount of DGPP phosphatase activity (1.3 units/mg) was exhibited with intact vacuoles (Fig. 5). For this experiment, the concentrations of DGPP and Triton X-100 were reduced from the standard assay conditions to 0.04 and 0.4 mM (0.024%), respectively. Under these conditions, the concentration of detergent was high enough to solubilize the phospholipid substrate delivered to the assay system but was not high enough to rupture the vacuole. These results indicated that the enzyme catalytic site was oriented to the cytosolic face of the vacuole membrane. Vacuole membranes were then ruptured with Triton X-100 and assayed for DGPP phosphatase activity. For this experiment, the concentrations of DGPP and Triton X-100 were 0.04 and 8 mM (0.4%), respectively. The activity (7 units/mg) found in detergent-solubilized vacuoles was 5-fold greater than that observed with the intact vacuoles (Fig. 5).

To further examine whether the catalytic site of DGPP phosphatase was exposed to the cytosolic face of the vacuole, enzyme activity was measured with intact vacuoles in an assay system that did not utilize Triton X-100 to solubilize the DGPP substrate. Instead, α-32P-labeled DGPP was delivered to the assay in phospholipid vesicles. Following the reaction, the phospholipid vesicles were separated from intact vacuoles by centrifugation. Phospholipids were extracted with chloroform and analyzed by thin-layer chromatography. The DGPP phosphatase in the intact vacuoles catalyzed a time-dependent conversion of DGPP to PA (Fig. 6). Vacuoles derived from the dpp1Δ mutant expressing the vector control did not exhibit DGPP phosphatase activity (Fig. 6).

Peptide Analysis of DGPP Phosphatase After the Treatment of Vacuoles with Endoproteinase Lys-C—There are six lysine
residues found in the DGPP phosphatase enzyme. The lysine residues at the N-terminal (Lys⁸ and Lys¹⁶) and C-terminal (Lys²⁵⁷) ends are located on one side of the membrane, whereas the lysine residues in loop L2 (Lys¹¹⁸, Lys¹⁴⁷, and Lys¹⁵₃) are on the other side of the membrane (Fig. 7A). We analyzed DGPP phosphatase peptides that were produced from intact vacuoles after treatment with endoproteinase Lys-C, a protease that cleaves the carboxyl side of peptide bonds of lysine residues. In the first set of experiments, peptides were analyzed by Western blotting using anti-HA antibodies to detect fragments containing the N-terminal HA epitope. If loop L2 faces the cytosolic side of the vacuole, we would expect that peptide fragments of 19, 18.4, and 15 kDa would be generated. On the other hand, if the N- and C-terminal lysine residues face the cytosolic side of the vacuole, peptide fragments of 31, 2.9, and 2.1 kDa would be generated. Immuno blot analysis showed that 18- and 15-kDa peptides were produced from intact vacuoles by digestion with endoproteinase Lys-C (Fig. 7B, left). This result suggested that loop L2 was on the cytosolic face of the intact vacuole. Endoproteinase Lys-C digestion of detergent-solubilized vacuoles resulted in a great reduction of the HA-containing peptides (Fig. 7B, left). As indicated above, cleavage of DGPP phosphatase at Lys⁸ or Lys¹⁶ would produce 2- or 3-kDa peptides containing an HA epitope. However, these fragments were not detected in our immunoblot analysis, probably because of poor retention of the peptides on the membrane during the electrophoretic transfer. Attempts to solve this problem by using membranes with smaller pore sizes were unsuccessful. The striking reduction of the HA-containing peptides after endoproteinase Lys-C treatment of detergent-solubilized vacuoles suggested that the N-terminal lysine residues were localized in the vacuole lumen.

The peptides derived from endoproteinase Lys-C treatment of intact vacuoles were analyzed with antibodies directed against the C-terminal epitope of DGPP phosphatase. Immunoblot analysis revealed that C-terminal peptides of 19 and 16 kDa were produced by the protease treatment (Fig. 7B, right). This was consistent with the cleavage of Lys¹¹⁸ and Lys¹⁴⁷ (and/or Lys¹⁵₃) in loop L2 and that these residues faced the cytosolic side of the intact vacuole. If the N- and C-terminal lysine residues faced the cytosolic side of the vacuole, peptide

Fig. 5. DGPP phosphatase activity exhibited by intact and ruptured vacuoles. DGPP phosphatase activity was measured with 50 ng of intact and ruptured vacuoles using 0.04 mm [γ-32P]DGPP in Triton X-100 mixed micelles. The final concentrations of Triton X-100 in the assay systems for the intact and ruptured vacuoles were 0.4 mm (0.02%), and 0.04% (0.4%), respectively. Each data point represents the average of triplicate determinations ± S.D. The integrity of the intact vacuoles was monitored by staining with fluorescent dye FM4-64.
...fragments of 32, 31, and 4 kDa would have been generated. Interestingly, the peptides produced from detergent-solubilized vacuoles were the same size as those produced from intact vacuoles (Fig. 7B, right). This result indicated that the lysine residues (Lys200 and Lys257) in the C-terminal region of DGPP phosphatase were less susceptible to endoproteinase Lys-C digestion.

A 20-kDa peptide was detected in the detergent-solubilized vacuoles with the anti-HA antibodies that were not treated with endoproteinase Lys-C (Fig. 7B, left). The HA-containing peptide was presumably generated from an endogenous vacuole protease whose identity was unknown. Peptide(s) containing the C-terminal epitope were not detected under the same condition (Fig. 7B, right). This may be attributed to the loss of the C-terminal epitope by proteolytic cleavage or to the generation of small peptides that were not detected by immunoblot analysis.

Cysteine Labeling of DGPP Phosphatase—DGPP phosphatase contains three cysteine residues in the central region of the protein. Cys132 and Cys150 are located in hydrophilic loop L2 and Cys185 is located in the fourth transmembrane region (Fig. 8A). The asymmetric distribution of these cysteine residues permitted the examination of the topography of the DGPP phosphatase using the sulfhydryl reagents MPB and AMS (41). The biotinylated reagent MPB is permeable to membranes, whereas the AMS reagent is impermeable to membranes (41).

The presence of the multiple DGPP phosphatase bands was due to endogenous proteolytic cleavage.

Effect of Zinc on Vacuole Membrane Phospholipid Composition—DGPP has been identified in total cellular extracts from S. cerevisiae (5, 8, 20, 35). However, it has not been identified in vacuole membranes where DGPP phosphatase is localized. To...
Vacuoles were isolated from wild type cells grown in the presence and absence of zinc. Phospholipids were extracted from vacuoles, separated by two-dimensional thin-layer chromatography, and analyzed for phosphate content as described under "Experimental Procedures." Each data point represents the average of two independent vacuole preparations ± S.D.

address this issue, phospholipids were extracted from vacuoles of wild type cells and analyzed by two-dimensional thin-layer chromatography as described under "Experimental Procedures." DGPP was present in the vacuolar membrane and accounted for 0.6 mol % of the total vacuolar membrane phospholipids (Fig. 9, inset). As described previously (53), the major vacuolar membrane phospholipids included PC, PE, PI, PS, and PA (Fig. 9).

Previous studies have shown that zinc availability regulates the expression of the DPP1-encoded DGPP phosphatase (4). Under zinc-depleted conditions, the transcription factor Zap1p binds the DPP1 promoter and induces the expression of DGPP phosphatase (4). Accordingly, we questioned whether zinc availability affected the level of DGPP in vacuole membranes. Vacuoles were isolated from cells grown in the presence and absence of zinc and analyzed for phospholipid composition. Zinc depletion resulted in a decrease in DGPP content from 0.6 mol % to an undetectable level (Fig. 9, inset). This finding was consistent with the increase in expression of DGPP phosphatase activity in response to zinc depletion (4). PA, which is also a substrate for the DGPP phosphatase enzyme (5), was also affected by zinc depletion. The amount of PA in vacuoles of zinc-supplemented cells (1.4 mol %) was reduced (79%) to 0.3 mol % in vacuoles isolated from zinc-depleted cells (Fig. 9, inset). The depletion of zinc from the growth medium also affected the levels of major vacuolar membrane phospholipids (Fig. 9). When compared with the vacuoles of cells grown in the presence of zinc, the level of PE decreased by 36%, whereas the levels of PI and PS increased by 44 and 29%, respectively.

DISCUSSION

The DPP1-encoded DGPP phosphatase of S. cerevisiae has been purified to homogeneity and characterized with respect to its enzymological and kinetic properties (2, 4–7, 20, 25). The enzyme catalyzes the dephosphorylation of DGPP to yield PA and then catalyzes the dephosphorylation of PA to yield diacylglycerol (5). The metabolism of DGPP in plants plays a role in lipid signaling under conditions of stress (14, 15, 15–17), and the substrate and products of the DGPP phosphatase have lipid messenger roles in mammalian cells (23, 54, 55). The expression of the yeast DPP1 gene is induced by the stress conditions of stationary phase and zinc depletion and by inositol supplementation (4, 20). The DGPP phosphatase is localized to the vacuole membrane in the cell (4). Its subcellular location suggests that the DGPP phosphatase might play a role in vacuole function under stress conditions by regulating vacuole membrane pools of DGPP and PA. Our initial approach in addressing this hypothesis was to examine the topography of the DGPP phosphatase catalytic site.

A significant amount of DGPP phosphatase activity was observed in intact vacuoles. This suggested that the catalytic site of the enzyme faced the cytosol. However, the apparent latent enzyme activity observed in detergent-solubilized vacuoles raised the question of whether the vacuole contained a population of enzyme with its catalytic site facing the lumen of the vacuole. Alternatively, the latent activity may result from detergent-mediated stimulation of the enzyme that contains its catalytic site exposed to the cytosol. We favored the latter explanation because of the fact that pure DGPP phosphatase (5) is one of several phospholipid-dependent enzymes that is stimulated by Triton X-100 and exhibits surface dilution kinetics (56). Moreover, the solubilization of the enzyme (5) from the vacuole membrane may have activated the enzyme by increasing enzyme-substrate accessibility (56, 57).

Protease protection analysis and chemical labeling have been used to study the topography of membrane-associated proteins (41, 58–60). We utilized these methods to further examine the topography of the DGPP phosphatase catalytic site. Digestion with endoprotease Lys-C and labeling of cysteine residues with sulfhydryl reagents substantiated the conclusion that the catalytic site of DGPP phosphatase was located on the cytosolic face of the vacuole membrane. The sizes of the N- and C-terminal peptides derived from intact and ruptured vacuoles after treatment with endoprotease Lys-C were consistent with the topographical model of the DGPP phosphatase catalytic site facing the cytosol. Moreover, the overall labeling of the cysteine residues of the enzyme in the intact vacuoles was strongly diminished by the membrane-impermeable sulfhydryl reagent (AMS) that blocks the MPB labeling of cysteine residues on the cytosolic face of the vacuole. The topography of the N and C termini was not defined directly because of the small size of HA-containing cleavage products and because of reduced efficiency of cleavage at the C-terminal end of the protein.

The topography of the yeast DGPP phosphatase catalytic site differs from that of the closely related lipid phosphate phosphatase enzymes of mammalian cells (24, 61). These lipid phosphatase family members are primarily localized to the plasma membrane, and their catalytic sites are oriented to the external leaflet of the membrane (24, 61). The mammalian lipid phosphatases also differ by their N-linked glycosylation on the external leaflet of transmembrane regions three and four on the external leaflet of the membrane (24, 61). Studies with various cell types indicate that this orientation is important for the degradation of bioactive lipid phosphate molecules (e.g., lyso-PA) in both the extracellular space and outer leaflet of the plasma membrane (23, 24, 61–64). The topography of the yeast DGPP phosphatase catalytic site suggests that the enzyme functions to regulate the levels of DGPP and PA on the leaflet of the vacuole membrane exposed to the cytosol. Our enzyme activity data using DGPP incorporated into phospholipid vesicles also indicated that the enzyme could metabolize DGPP in membrane structures external to the vacuole.

The functional role of the DGPP phosphatase enzyme in the vacuole is supported by the presence of its substrates in the vacuole membrane. DGPP and PA accounted for 0.6 and 1.4 mol %, respectively, of the total vacuolar membrane phospholipids. DGPP was not identified as a vacuole membrane phospholipid in the earlier work of Zinser et al. (53). The fact that DGPP is a recently discovered phospholipid in S. cerevisiae (5) suggests that it may have been grouped within a relatively large percentage of unidentified vacuole membrane phospholipids labeled as "others" in this earlier work (53). Interestingly, the
amount of DGPP diminished to an undetectable level when cells were depleted for zinc, a stress condition that greatly induces (10-fold) DGPP phosphatase expression (4). Zinc depletion also caused a significant reduction (79%) in the amount of PA in the vacuole membrane. This was also consistent with the induction of DGPP phosphatase under this stress condition. Along with changes in DGPP and PA, zinc depletion resulted in changes of the amount of major vacuole membrane phospholipids PE, PI, and PS. Upon zinc depletion, there was a 38% decrease in PE and 44 and 29% increases in PI and PS, respectively. These changes, however, were not dependent on the regulation of DPP1 gene expression. Similar changes in PE, PI, and PS composition are also exhibited in the vacuoles of dpp1Δ mutant cells depleted for zinc.2 Instead, changes in these phospholipids can be attributed to zinc-mediated regulation of other enzymes of phospholipid metabolism.

The yeast vacuole is an acidic organelle (analogous to the mammalian lysosome) that contains a cadre of hydrolytic enzymes required for turnover of macromolecules as well as cellular organelles (i.e. autophagy) (65–67). It is also a reservoir for transporting ions, and polyphosphates (65, 66). Vacuole function is important during times of stress when cells respond to nutrient limitation by recycling cellular materials (65–69). The pathways that organelles, proteins, and small molecules follow to the vacuole are well characterized (65–69). The pathways involved in autophagy are also induced (30). Whether the removal of DGPP in the vacuole membrane is related to the process of autophagy or other vesicle transport processes is unknown. The studies reported here provide a foundation for addressing these questions.

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