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Purification and Characterization of Diacylglycerol Pyrophosphate Phosphatase from *Saccharomyces cerevisiae**

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Diacylglycerol pyrophosphate (DGPP) phosphatase is a novel membrane-associated enzyme that catalyzes the dephosphorylation of the β phosphate of DGPP to yield phosphatidate and P_i . DGPP phosphatase was purified 33,333-fold from *Saccharomyces cerevisiae* by a procedure that included Triton X-100 solubilization of microsomal membranes followed by chromatography with DE53, Affi-Gel Blue, hydroxylapatite, and Mono Q. The procedure resulted in the isolation of an apparent homogeneous protein with a subunit molecular mass of 34 kDa. DGPP phosphatase activity was associated with the 34-kDa protein. DGPP phosphatase had a broad pH optimum between 6.0 and 8.5 and was dependent on Triton X-100 for maximum activity. The enzyme was inhibited by divalent cations, NaF, and pyrophosphate and was relatively insensitive to thioreactive agents. The turnover number (molecular activity) for the enzyme was $5.8 \times 10^3 \text{ min}^{-1}$ at pH 6.5 and 30 °C. DGPP phosphatase exhibited typical saturation kinetics with respect to DGPP ($K_m = 0.55 \text{ mol } \%$). The K_m value for DGPP was 3-fold greater than its cellular concentration (0.18 mol %). DGPP phosphatase also catalyzed the dephosphorylation of phosphatidate, but this dephosphorylation was subsequent to the dephosphorylation of the β phosphate of DGPP. The dependence of activity on phosphatidate ($K_m = 2.2 \text{ mol } \%$) was cooperative (Hill number = 2.0). DGPP was the preferred substrate for the enzyme with a specificity constant (V_{\max} / K_m) 10-fold greater than that for phosphatidate. In addition, DGPP potently inhibited ($K_i = 0.35 \text{ mol } \%$) the dephosphorylation of phosphatidate by a competitive mechanism whereas phosphatidate did not inhibit the dephosphorylation of DGPP. DGPP was neither a substrate nor an inhibitor of pure phosphatidate phosphatase from *S. cerevisiae*. DGPP was synthesized from phosphatidate via the phosphatidate kinase reaction.

Diacylglycerol pyrophosphate (DGPP)¹ is a novel phospholipid metabolite recently identified from the plant *Catharanthus roseus* by Wissing and Behrbohm (1). DGPP contains a pyrophosphate group attached to diacylglycerol (Fig. 1). This compound was previously observed by several workers (2–4) as the phospholipid product of a lipid kinase reaction in plants that was not identified correctly (1). It is now known that DGPP is synthesized from PA and ATP through the reaction catalyzed by the novel enzyme PA kinase (1). PA kinase is a ubiquitous membrane-associated enzyme found in the plant kingdom (5). The enzyme has been purified from suspension-cultured *C. roseus* cells (5) and characterized with respect to its enzymological and kinetic properties (6).

Metabolic labeling studies using *C. roseus* have shown that DGPP is rapidly metabolized by a membrane-associated phosphatase, which has been named DGPP phosphatase.² DGPP phosphatase catalyzes the dephosphorylation of DGPP to form PA and P_i .² In addition to being present in plant cells, DGPP phosphatase activity is also present in membrane fractions of *Escherichia coli*, *Saccharomyces cerevisiae*, and rat liver.² Whereas it is unclear what role DGPP plays in phospholipid metabolism and cell growth, PA, the product of the DGPP phosphatase reaction, plays a major role in lipid metabolism. PA is the precursor of all phospholipids and triacylglycerol (7, 8). In addition, PA regulates the activity of several lipid-dependent enzymes (9–12) and has mitogenic effects in animal cells (13–15). Thus, the discovery of DGPP phosphatase activity in a wide range of organisms suggests that this enzyme may play an important role in phospholipid metabolism and cell growth.

A purified preparation of DGPP phosphatase is required for defined studies on the mechanism and regulation of this novel enzyme of phospholipid metabolism. Owing to its amenable molecular genetic system, we are using the yeast *S. cerevisiae* as a model eucaryote to study DGPP phosphatase. We report in this paper the purification of DGPP phosphatase to apparent homogeneity. The purified enzyme was characterized with respect to its enzymological and kinetic properties. Moreover, we demonstrated that *S. cerevisiae* synthesized DGPP via the PA kinase reaction.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were reagent grade. Growth medium supplies were purchased from Difco. Radiochemicals were from DuPont NEN. Scintillation counting supplies and acrylamide for electrophoresis were from

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¹ The abbreviations used are: DGPP, diacylglycerol pyrophosphate; PA, phosphatidate.

² B. Riedel, W.-I. Wu, G. M. Carman, and J. B. Wissing, manuscript in preparation.

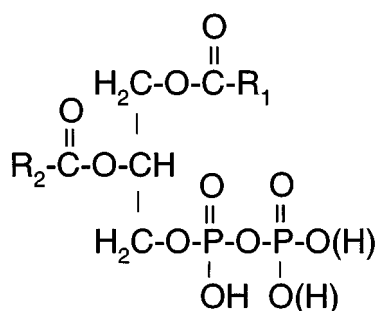


FIG. 1. Structure of DGPP.

National Diagnostics. Nucleotides, pyrophosphate, glycerol 3-phosphate, inositol 1-phosphate, *N*-ethylmaleimide, *p*-chloromercuriphenylsulfonic acid, phenylmethanesulfonyl fluoride, Triton X-100, and bovine serum albumin were purchased from Sigma. Phospholipids were purchased from Avanti Polar Lipids and Sigma. DE53 (DEAE-cellulose) was purchased from Whatman. Affi-Gel Blue, hydroxylapatite (Bio-Gel HT), molecular mass standards for SDS-polyacrylamide gel electrophoresis, electrophoresis reagents, and protein assay reagent were purchased from Bio-Rad. Mono Q was purchased from Pharmacia Biotech Inc. Silica gel 60 thin-layer chromatography plates were from EM Science. *E. coli* diacylglycerol kinase was obtained from Lipidex Inc.

Methods

Strain and Growth Conditions

Strain *MATa ade5* (16), which shows normal regulation of phospholipid metabolism (17–20), was used for the purification of DGPP phosphatase. Cultures were maintained on YEPD medium (1% yeast extract, 2% peptone, 2% glucose) plates containing 2% Bacto-agar. For enzyme purification, cells were grown in YEPD medium at 30 °C to late exponential phase, harvested by centrifugation, and stored at –80 °C as described previously (21).

Preparation of Enzymes

PA kinase was purified from suspension-cultured *C. roseus* cells as described by Wissing and Behrbohm (5). PA phosphatase was purified from *S. cerevisiae* as described by Lin and Carman (22). The total membrane fraction (22) of *S. cerevisiae* was used for the assay of PA kinase activity.

Preparation of DGPP Standard and Labeled Substrates

DGPP standard was synthesized enzymatically from PA and ATP using purified *C. roseus* PA kinase as described by Wissing and Behrbohm (1). Purified PA kinase was also used to prepare ^{32}P -labeled DGPP. [α - ^{32}P]DGPP was synthesized from [^{32}P]PA and ATP. [β - ^{32}P]DGPP was prepared from PA and [γ - ^{32}P]ATP. ^{32}P -Labeled DGPP was 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). [^{32}P]PA was synthesized from diacylglycerol and [γ - ^{32}P]ATP using *E. coli* diacylglycerol kinase (23). Labeled PA was purified by thin-layer chromatography using the solvent system chloroform/methanol/water (65:25:4).

Electrophoresis

Polyacrylamide gel electrophoresis under nondenaturing conditions (24) was performed at 5 °C in 6% slab gels containing 0.5% Triton X-100. SDS-polyacrylamide gel electrophoresis (25) was performed with 9% slab gels. Molecular mass standards were phosphorylase *b* (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa). Proteins on SDS-polyacrylamide gels were stained with silver (26).

Enzyme Assays and Product Identification

The optimal conditions for assay of DGPP phosphatase activity were determined in detail with purified enzyme. DGPP phosphatase activity was measured by monitoring the release of water-soluble $^{32}\text{P}_i$ from 0.1 mM [β - ^{32}P]DGPP (10,000–20,000 cpm/nmol) in 50 mM Tris-maleate buffer (pH 6.5) containing 2 mM Triton X-100, 10 mM 2-mercaptoethanol, and enzyme protein in a total volume of 0.1 ml. Purified DGPP phosphatase was routinely diluted 2,000-fold for enzyme assays. The reaction was terminated by addition of 0.5 ml of 0.1 N HCl in methanol. Chloroform (1 ml) and 1 M MgCl₂ (1 ml) were added, the system was

mixed, and the phases were separated by 2 min of centrifugation at 100 × *g*. Ecoscint H (4 ml) was added to a 0.5-ml sample of the aqueous phase, and radioactivity was determined by scintillation counting. Alternatively, DGPP phosphatase activity was measured by monitoring the formation of [^{32}P]PA from [α - ^{32}P]DGPP (10,000–20,000 cpm/nmol) under the assay conditions described above. The chloroform-soluble phospholipid product of the reaction, PA, was analyzed with standard PA and DGPP 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). The positions of the labeled phospholipids on the chromatograms were determined by autoradiography. The amount of labeled phospholipids was determined by scintillation counting.

PA kinase activity was measured with 40 mM imidazole-HCl buffer (pH 6.1), 10 mM MgCl₂, 100 mM NaCl, 0.5 mM dithiothreitol, 10 mM NaF, 5 mM β -glycerol phosphate, 0.1 mM MnCl₂, 6.9 mM Triton X-100, 0.6 mM PA, 1 mM [γ - ^{32}P]ATP (100,000 cpm/nmol), and 0.3 mg/ml membrane protein (1, 5). NaF, β -glycerol phosphate, and MnCl₂ were included in the reaction mixture to inhibit phosphatase reactions. The ^{32}P -labeled chloroform-soluble phospholipid product of the reaction, DGPP, was analyzed with standard DGPP by thin-layer chromatography as described above for the DGPP phosphatase assay.

PA phosphatase (3-*sn*-phosphatidate phosphohydrolase, EC 3.1.3.4) was measured with 50 mM Tris maleate buffer (pH 7.0), 10 mM 2-mercaptoethanol, 2 mM MgCl₂, 1 mM Triton X-100, 0.1 mM [^{32}P]PA, and enzyme protein (27). A unit of enzymatic activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product/min unless otherwise indicated. Specific activity was defined as units/mg of protein.

Protein Determination

Protein concentration was determined by the method of Bradford (28) using bovine serum albumin as the standard. Buffers that were identical to those containing protein samples were used as blanks. Protein concentration of samples after Mono Q chromatography was determined by scanning densitometry of silver-stained SDS-polyacrylamide gels using bovine serum albumin as the standard. Protein was monitored during purification on columns by measuring the absorbance at 405 nm.

Preparation of Triton X-100/Phospholipid-mixed Micelles

Phospholipids in chloroform were transferred to a test tube, and solvent was removed *in vacuo* for 40 min. Triton X-100/phospholipid-mixed micelles were prepared by adding Triton X-100 to dried lipids. The total phospholipid concentration in Triton X-100/phospholipid-mixed micelles did not exceed 15 mol % to ensure that the structure of the mixed micelles were similar to the structure of pure Triton X-100 (29, 30).

Analysis of Kinetic Data

Kinetic data were analyzed according to the Michaelis-Menten and Hill equations using the EZ-FIT enzyme kinetic model fitting program (31). EZ-FIT uses the Nelder-Mead Simplex and Marquardt/Nash nonlinear regression algorithms sequentially and tests for the best fit of the data among different kinetic models.

Mass Analyses of DGPP and PA

Phospholipids were extracted from cells using method IIIB described by Hanson and Lester (32) and dried *in vacuo*. The solvent mixture used in method IIIB is 95% ethanol/water/diethylether/pyridine/ammonium hydroxide (15:15:5:1:0.018). The sample was dissolved in chloroform/methanol/water (15:15:5). Samples were subjected to analytical normal phase high performance liquid chromatography using a 5- μm Kromasil silica column (150 × 4.6 mm, inner diameter) equilibrated with chloroform/methanol/30% ammonium hydroxide (80:19.5:0.5). Elution was carried out using a 14-min linear gradient (0–30%) of methanol/water/30% ammonium hydroxide (80:19.5:0.5) followed by holding the same solvent at 30% for an additional 14 min. The identity of DGPP was determined by comparing its elution profile with that of authentic DGPP (1) using an evaporative light scattering detector. A flow rate of 1 ml/min was used throughout. The cellular concentration of DGPP was calculated relative to the concentration of the major phospholipids in the extract. The cellular concentration of PA was determined as described previously (33).

Purification of DGPP Phosphatase

All steps were performed at 5 °C.

Step 1: Preparation of Cell Extract—The cell extract was prepared

TABLE I
Purification of DGPP phosphatase

DGPP phosphatase was purified from *S. cerevisiae* as described under "Experimental Procedures." The data are based on starting with 200 g (wet weight) of cells.

Purification step	Total units	Protein	Specific activity	Yield	Purification
	$\mu\text{mol}/\text{min}$	mg	units/mg	%	-fold
1. Cell extract	49.4	10,957.6	0.0045	100	1
2. Microsomes	27.4	2,312.8	0.0118	55.5	2.6
3. Triton X-100	16.2	803.2	0.0201	32.8	4.5
4. DE53	5.6	15.41	0.363	11.3	80.6
5. Affi-Gel Blue	5.5	2.62	2.10	11.1	466.6
6a. Hydroxylapatite (peak I)	0.51	0.143	3.49	1.0	775.5
6b. Hydroxylapatite (peak II)	2.36	0.185	12.7	4.7	2,822
7. Mono Q I	0.36	0.0024	150	0.73	33,333
8. Mono Q II	2.0	0.018	111	4.0	24,666

from 200 g (wet weight) of cells by disruption with glass beads with a Bead-Beater (Biospec Products) in buffer A (50 mM Tris-maleate (pH 7.0), 1 mM Na_2EDTA , 0.3 M sucrose, 10 mM 2-mercaptoethanol, and 0.5 mM phenylmethanesulfonyl fluoride) as described previously (21). Unbroken cells and glass beads were removed by centrifugation at $1,500 \times g$ for 5 min.

Step 2: Preparation of Microsomes—Microsomes were isolated from the cell extract by differential centrifugation (21). Microsomes were washed and resuspended in buffer B (50 mM Tris-maleate (pH 7.0), 10 mM MgCl_2 , 10 mM 2-mercaptoethanol, 20% glycerol, and 0.5 mM phenylmethanesulfonyl fluoride). These membranes were routinely frozen at -80°C until used for purification.

Step 3: Preparation of Triton X-100 Extract—Microsomes were suspended in buffer B containing 1% Triton X-100 at a final protein concentration of 10 mg/ml. The suspension was incubated for 1 h on a rotary shaker at 150 rpm. After the incubation, the suspension was centrifuged at $100,000 \times g$ for 1.5 h to obtain the Triton X-100 extract (supernatant).

Step 4: DE53 Chromatography—A DE53 column (2.5×20.5 cm) was equilibrated with buffer C (50 mM Tris-maleate (pH 7.0), 10 mM MgCl_2 , 10 mM 2-mercaptoethanol, 20% glycerol, and 1% Triton X-100). The Triton X-100 extract was applied to the column at a flow rate of 60 ml/h. The column was washed with one column volume of buffer C followed by elution of DGPP phosphatase activity in 6-ml fractions with nine column volumes of a linear NaCl gradient (0–0.25 M) in buffer C. The peak of DGPP phosphatase activity eluted from the column at the beginning of the NaCl gradient. The most active fractions containing activity were pooled and used for the next step in the purification scheme.

Step 5: Affi-Gel Blue Chromatography—An Affi-Gel Blue column (2.0×16 cm) was equilibrated with buffer C. DE53-purified enzyme was applied to the column at a flow rate of 30 ml/h. The column was washed with one column volume of buffer C followed by two column volumes of buffer C containing 0.3 M NaCl. DGPP phosphatase activity was eluted from the column in 3.5-ml fractions with 10 column volumes of a linear NaCl gradient (0.3–0.9 M) in buffer C. The peak of DGPP phosphatase activity eluted from the column at a NaCl concentration between 0.3 and 0.4 M. The most active fractions were pooled and the enzyme preparation was desalted by dialysis against buffer D (10 mM potassium phosphate (pH 7.0), 10 mM MgCl_2 , 10 mM 2-mercaptoethanol, 20% glycerol, and 1% Triton X-100).

Step 6: Hydroxylapatite Chromatography—A hydroxylapatite column (1.5×8.5 cm) was equilibrated with buffer D. The desalted Affi-Gel Blue-purified enzyme was applied to the column at a flow rate of 20 ml/h. The column was washed with one column volume of buffer D, and DGPP phosphatase activity was eluted from the column in 2-ml fractions with 20 column volumes of a linear potassium phosphate gradient (10–150 mM) in buffer D. Two peaks of DGPP phosphatase activity eluted from the column. The first peak (peak I) of activity eluted from the column at the beginning of the gradient. The second peak (peak II) of activity eluted at a phosphate concentration of about 24 mM. The most active fractions from each peak were pooled and dialyzed against buffer C.

Step 7: Mono Q I Chromatography—A Mono Q column (0.5×5 cm) was equilibrated with buffer C. Hydroxylapatite-purified enzyme from peak I was applied to the column at a flow rate of 24 ml/h. The column was washed with two column volumes of buffer C. DGPP phosphatase activity was eluted from the column in 1-ml fractions with 40 column volumes of a linear NaCl gradient (0–0.3 M) in buffer C. The peak of DGPP phosphatase activity eluted from the column at a NaCl concentration of about 0.12 M. Fractions containing activity were pooled and

stored at -80°C . The purified enzyme was completely stable for at least 2 months.

Step 8: Mono Q II Chromatography—A second Mono Q column (0.5×5 cm) was equilibrated with buffer C. Hydroxylapatite-purified enzyme from peak II was applied to the column at a flow rate of 24 ml/h. The column was washed with two column volumes of buffer C. DGPP phosphatase activity was eluted from the column in 1-ml fractions with 40 column volumes of a linear NaCl gradient (0–0.3 M) in buffer C. The peak of DGPP phosphatase activity eluted from the column at the beginning of the NaCl gradient. Fractions containing activity were pooled and stored at -80°C . The purified enzyme was completely stable for at least 2 months.

RESULTS

Purification of DGPP Phosphatase—DGPP phosphatase activity was associated with the microsomal fraction of the cell. No activity was associated with the cytosolic fraction. DGPP phosphatase activity could not be dissociated from membranes by treatment with buffers containing high ionic strength. Therefore, the nature of the association of DGPP phosphatase activity with membranes was integral. A summary of the purification of DGPP phosphatase from microsomes is shown in Table I. DGPP phosphatase activity was solubilized from the microsomal fraction with 1% Triton X-100 followed by chromatography with DE53, Affi-Gel Blue, hydroxylapatite, and Mono Q. The hydroxylapatite chromatography step resulted in the isolation of two distinct peaks of DGPP phosphatase activity (Fig. 2A). Fractions containing activity under each of the peaks were further purified on separate Mono Q columns. Hydroxylapatite peak I DGPP phosphatase activity eluted from Mono Q I at a salt concentration of 0.12 M (Fig. 2B), whereas hydroxylapatite peak II DGPP phosphatase activity eluted from Mono Q II at the beginning of the salt gradient (Fig. 2C). Mono Q I chromatography of hydroxylapatite peak I enzyme resulted in the isolation of an apparent homogeneous protein preparation as shown by SDS-polyacrylamide gel electrophoresis (Fig. 2B). The minimum subunit molecular mass of the purified protein was 34 kDa. Mono Q II chromatography of hydroxylapatite peak II DGPP phosphatase resulted in the isolation of a protein preparation that contained a major protein doublet migrating at a molecular mass of 34 kDa (Fig. 2C). This DGPP phosphatase preparation also contained some minor protein contaminants (Fig. 2C). Overall, the hydroxylapatite peak I DGPP phosphatase was purified 33,333-fold over the cell extract with an activity yield of 0.73% to a final specific activity of 150 $\mu\text{mol}/\text{min}/\text{mg}$ (Table I). Hydroxylapatite peak II DGPP phosphatase was purified 24,666-fold over the cell extract to a final specific activity of 111 $\mu\text{mol}/\text{min}/\text{mg}$ with an activity yield of 4% (Table I).

To confirm that the 34-kDa protein in the purified DGPP phosphatase preparations was indeed DGPP phosphatase, we used combination of native and SDS-polyacrylamide gel electrophoresis and measuring activity from gel slices (34). Purified

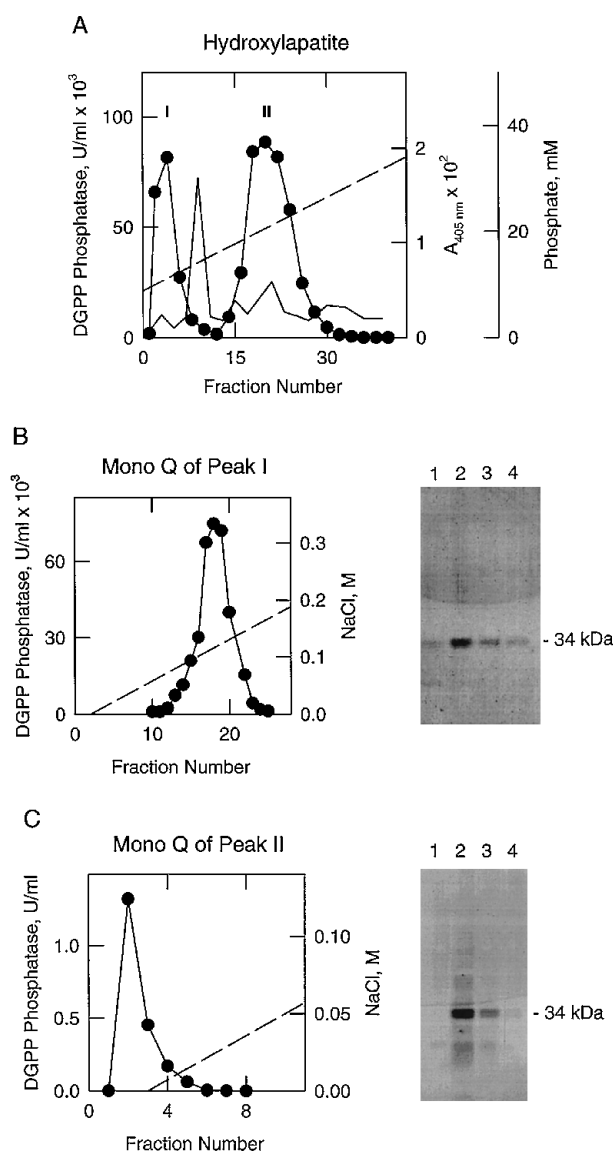


FIG. 2. Elution profiles of DGPP phosphatase activity after chromatography with hydroxylapatite, Mono Q I, and Mono Q II and SDS-polyacrylamide gel electrophoresis of purified enzyme. DGPP phosphatase was subjected to hydroxylapatite chromatography (panel A). Fractions containing DGPP phosphatase activity under peaks I and II were subjected to chromatography with Mono Q I (panel B) and Mono Q II (panel C), respectively. Fractions were collected and assayed for DGPP phosphatase activity (●) and protein (—). Phosphate (panel A) and NaCl (panels B and C) gradient profiles are indicated by a dashed line. The protein concentration in the fractions from the Mono Q columns was too low to be detected spectrophotometrically. Fractions 15 and 16 (lane 1), 17 and 18 (lane 2), 19 and 20 (lane 3), and 21 and 22 (lane 4) from the Mono Q I column (panel B) were subjected SDS-polyacrylamide gel electrophoresis (right of figure). Fractions 1 (lane 1), 2 (lane 2), 3 (lane 3), and 4 (lane 4) from the Mono Q II column (panel C) were subjected SDS-polyacrylamide gel electrophoresis (right of figure). The position of the 34-kDa subunit of DGPP phosphatase is indicated in the figures shown in panels B and C.

enzyme was subjected to native polyacrylamide gel electrophoresis in the presence of 0.5% Triton X-100 at 5 °C. Following electrophoresis, one lane from a slab gel was stained with silver and showed a single protein band with an R_F of 0.6. The protein from the native gel was excised, denatured, and subjected to SDS-polyacrylamide gel electrophoresis. This analysis showed a 34-kDa protein band. A second lane was cut into 0.5-cm slices, and each slice was minced with a razor blade in assay buffer and homogenized at 5 °C. The samples were then assayed for DGPP phosphatase activity using $[\beta\text{-}^{32}\text{P}]\text{DGPP}$ as substrate.

DGPP phosphatase activity was found in those polyacrylamide gel slices which contained the protein migrating with an R_F of 0.6. The counts of water-soluble $^{32}\text{P}_i$ hydrolyzed from $[\beta\text{-}^{32}\text{P}]\text{DGPP}$ were 50,000–60,000 cpm above the counts derived from gel slices not containing protein. We were unable to measure DGPP phosphatase activity directly from SDS-polyacrylamide gel slices. Since the enzyme purified from hydroxylapatite peak I was essentially pure and had a higher specific activity than the enzyme purified from peak II, it was used for the characterization of DGPP phosphatase activity.

Time Dependence of DGPP Phosphatase Activity and Identification of Reaction Products—A standard DGPP phosphatase reaction was carried out using 10 nmol of $[\beta\text{-}^{32}\text{P}]\text{DGPP}$ as substrate. The hydrolysis of $^{32}\text{P}_i$ from DGPP was linear for 2 h (Fig. 3A). By the time the reaction went to completion (5 h), 80% of the substrate was converted to the product P_i (Fig. 3A). To confirm that the other product of the DGPP phosphatase reaction was PA, $[\alpha\text{-}^{32}\text{P}]\text{DGPP}$ was used as the substrate. The chloroform-soluble product of the reaction was analyzed by thin-layer chromatography. DGPP phosphatase catalyzed a time-dependent conversion of DGPP to PA (Fig. 3B). Furthermore, these results showed that DGPP phosphatase catalyzed the hydrolysis of the β phosphate of DGPP as opposed to the hydrolysis of the pyrophosphate moiety of DGPP. The water-soluble fraction of the reaction was also analyzed. After an initial lag period, DGPP phosphatase catalyzed a time-dependent hydrolysis of the α phosphate from $[\alpha\text{-}^{32}\text{P}]\text{DGPP}$ (Fig. 3C). This result indicated that DGPP phosphatase could also use PA as a substrate (see below). Under the standard reaction time (10–20 min) used for the DGPP phosphatase assay, very little of the α phosphate of DGPP would be hydrolyzed by the enzyme (Fig. 3C).

Effect of pH, Divalent Cations, and Triton X-100 on DGPP Phosphatase Activity—The effect of pH on DGPP phosphatase activity is shown in Fig. 4A. DGPP phosphatase exhibited a broad pH optimum between pH 6.0 and 8.5. Since activity was slightly higher at pH 6.5, activity was routinely measured at this pH. DGPP phosphatase activity was measured in the presence of a variety of divalent cations (Fig. 4B). Activity was independent of any divalent cation requirement. The addition of Mn^{2+} ($\text{IC}_{50} = 58 \mu\text{M}$), Ca^{2+} ($\text{IC}_{50} = 0.56 \text{ mM}$), and Mg^{2+} ($\text{IC}_{50} = 17 \text{ mM}$) ions to the assay system resulted in a dose-dependent inhibition of DGPP phosphatase activity. The most inhibitory divalent cation was Mn^{2+} (Fig. 4B, inset). DGPP phosphatase activity was measured in the presence of various concentrations of Triton X-100 (Fig. 4C). The addition of Triton X-100 to the assay mixture stimulated DGPP phosphatase activity to a maximum at a concentration of 2 mM (molar ratio of Triton X-100 to DGPP of 20:1), followed by an apparent inhibition of activity at concentrations above 2 mM. These results are indicative of surface dilution kinetics (35).

Effectors of DGPP Phosphatase Activity—The effect of various compounds on DGPP phosphatase activity was examined using a DGPP concentration (0.5 mol %) near its K_m value (see below). In this manner, we could more readily observe inhibitory or stimulatory effects on enzyme activity. The effect of various compounds on DGPP phosphatase activity is summarized in Table II. DGPP phosphatase activity was inhibited by NaF, but was insensitive to Na_2EDTA . The thioreactive compound *p*-chloromercuriphenylsulfonic acid inhibited DGPP phosphatase activity by 41%, whereas the enzyme was relatively insensitive to *N*-ethylmaleimide. At a 10-fold higher molar concentration over that of DGPP, pyrophosphate and ADP inhibited DGPP phosphatase activity by 46% and 24%, respectively. ATP and compounds containing a single phosphate group did not affect DGPP phosphatase activity.

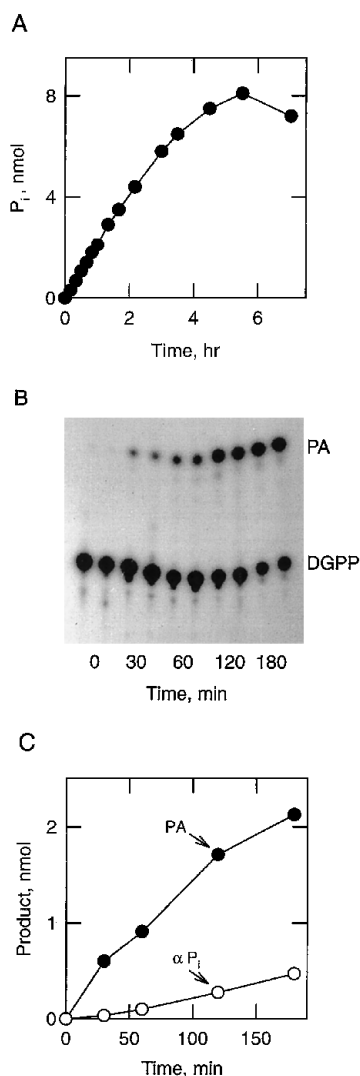


FIG. 3. Time dependence of DGPP phosphatase activity and identification of reaction products. *Panel A*, DGPP phosphatase activity was measured with 10 nmol of [β - 32 P]DGPP as substrate for the indicated time intervals. Following the incubations, the water-soluble 32 P, hydrolyzed from [β - 32 P]DGPP was analyzed by scintillation counting. *Panel B*, DGPP phosphatase activity was measured with 10 nmol of [α - 32 P]DGPP as substrate for the indicated time intervals. Following the incubations, the chloroform-soluble 32 P-labeled substrate and product of the reaction were analyzed by thin-layer chromatography. Duplicate lanes are shown for each time point in the figure. The positions of DGPP and PA standards are indicated. *Panel C*, DGPP phosphatase activity was measured with 10 nmol of [α - 32 P]DGPP as substrate for the indicated time intervals. Following the incubations, the chloroform-soluble 32 P-labeled product PA was separated from [α - 32 P]DGPP by thin-layer chromatography and then analyzed by scintillation counting. The water-soluble 32 P, hydrolyzed from [α - 32 P]DGPP was analyzed by scintillation counting.

The effect of the major *S. cerevisiae* phospholipids and diacylglycerol on DGPP phosphatase activity was examined. In these experiments, the surface concentration of lipids was 20-fold greater than that of DGPP. None of these lipids affected DGPP phosphatase activity by more than 50%. PA, the product of the reaction, did not affect activity.

Dependence of DGPP Phosphatase Activity on DGPP—Triton X-100/DGPP-mixed micelles were used in our kinetic experiments to examine the dependence of DGPP phosphatase activity on DGPP. This system permits the analysis of the enzyme in an environment that mimics the physiological surface of the membrane (35). In Triton X-100/DGPP-mixed micelles, DGPP phosphatase activity followed surface dilution kinetics (Fig.

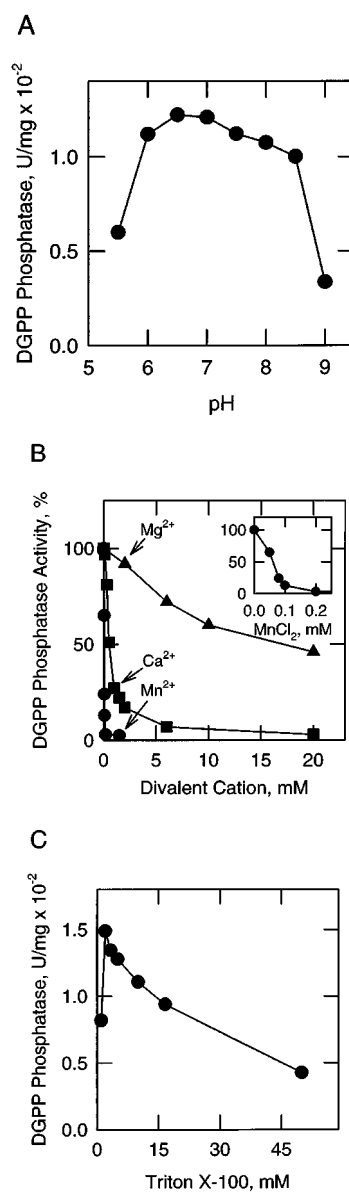


FIG. 4. Effect of pH, divalent cations, and Triton X-100 on DGPP phosphatase activity. DGPP phosphatase activity was measured at the indicated pH values with 50 mM Tris-maleate-glycine buffer (*panel A*); the indicated concentrations of $MgCl_2$, $CaCl_2$, and $MnCl_2$ (*panel B*); and the indicated concentrations of Triton X-100 (*panel C*).

4C). Thus, the concentration of DGPP in the mixed micelles was expressed as a surface concentration (in mol %) as opposed to a molar concentration (35). We examined the dependence of DGPP phosphatase activity on the surface concentration of DGPP using various set molar concentrations of DGPP. DGPP phosphatase activity exhibited typical saturation kinetics with respect to the surface concentration of DGPP (Fig. 5). Furthermore, the dependence of activity on DGPP surface concentration was independent of the molar concentrations of DGPP used in these experiments (Fig. 5). This was important to demonstrate because the results of the kinetic experiments would be difficult to interpret if activity was also dependent on the molar concentration of DGPP (35). The V_{max} was 172 μ mol/min/mg, and the K_m value for DGPP was 0.55 mol %. The turnover number (molecular activity) for the enzyme at pH 6.5 and 30 °C was calculated to be $5.8 \times 10^3 \text{ min}^{-1}$.

Dephosphorylation of PA by DGPP Phosphatase—DGPP phosphatase catalyzed the hydrolysis of the α phosphate of DGPP after the β phosphate of the substrate was hydrolyzed

TABLE II
Effectors of DGPP phosphatase activity

DGPP phosphatase activity was measured under standard assay conditions with 0.5 mol % DGPP (bulk concentration of 0.1 mM) in the presence of the indicated additions.

Component	Relative activity
	%
Control	100
+10 mM NaF	6
+2 mM Na ₂ EDTA	100
+1 mM <i>p</i> -chloromercuriphenylsulfonic acid	59
+5 mM <i>N</i> -ethylmaleimide	90
+1 mM pyrophosphate	54
+1 mM ADP	76
+1 mM ATP	93
+1 mM AMP	95
+1 mM glycerol 3-phosphate	101
+1 mM inositol 1-phosphate	100
+10 mol % phosphatidylcholine	106
+10 mol % phosphatidylethanolamine	124
+10 mol % phosphatidylinositol	139
+10 mol % phosphatidylserine	83
+10 mol % PA	105
+10 mol % diacylglycerol	89

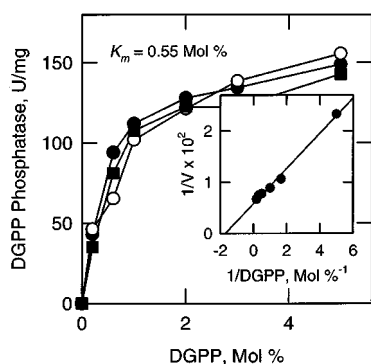


FIG. 5. **Dependence of DGPP phosphatase activity on the surface concentration of DGPP.** DGPP phosphatase activity was measured as a function of the surface concentration (mol %) of [β -³²P]DGPP. The molar concentration of DGPP was held constant at 0.1 mM (●), 0.15 mM (○), and 0.2 mM (■) while the Triton X-100 concentration was varied. The inset is a reciprocal plot of the data using DGPP at a molar concentration of 0.1 mM. The line drawn is the result of a least-squares analysis of the data.

(Fig. 3C). We addressed directly whether DGPP phosphatase could hydrolyze the phosphate group from PA. In these experiments, activity was measured under the optimal assay conditions for DGPP phosphatase. Indeed, DGPP phosphatase catalyzed the dephosphorylation of PA. We next examined the dependence of activity on the surface concentration of PA as described above for DGPP. The dependence of activity on the surface concentration of PA was cooperative (Fig. 6). The kinetics of the enzyme with respect to the surface concentration of PA was independent of the molar concentrations of PA used in these experiments (Fig. 6). Analysis of the data according to the Hill equation yielded a V_{max} of 70 $\mu\text{mol}/\text{min}/\text{mg}$, a K_m value for PA of 2.2 mol %, and a Hill number of 2.

We next examined the effect of DGPP on the ability of DGPP phosphatase to dephosphorylate PA. DGPP inhibited the dephosphorylation of PA in a dose-dependent manner (Fig. 7). A kinetic analysis was performed to examine the mechanism of DGPP inhibition on the activity using PA as the substrate. The dependence of activity on the surface concentration of PA was measured in the absence and presence of 0.5 mol % DGPP (Fig. 7, inset). The kinetic patterns of the enzyme reaction were cooperative (Hill number of 2.0) in the absence and presence of DGPP. The data were transformed to a double-reciprocal plot where the PA surface concentration was raised to the Hill

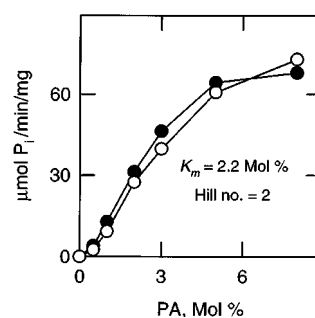


FIG. 6. **Kinetics of the dephosphorylation of PA by DGPP phosphatase.** Activity was measured as a function of the surface concentration (mol %) of [β -³²P]PA. The molar concentration of PA was held constant at 0.1 mM (●) and 0.2 mM (○) while the Triton X-100 concentration was varied.

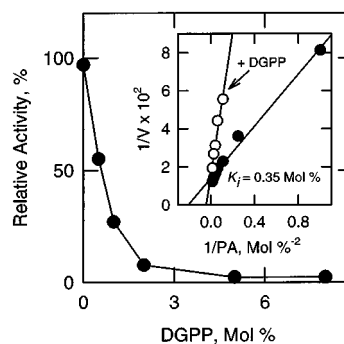


FIG. 7. **Effect of DGPP on the dephosphorylation of PA by DGPP phosphatase.** Activity was measured with 3 mol % [β -³²P]PA (molar concentration of 0.1 mM) as substrate in the presence of the indicated surface concentrations of DGPP. Inset, activity was measured as a function of the surface concentration (mol %) of [β -³²P]PA in the absence (●) and presence (○) of 0.5 mol % DGPP (molar concentration of 0.1 mM). The data are plotted as $1/V$ (units/mg) versus the reciprocal of the PA surface concentration raised to the Hill number of 2. The lines drawn are the result of a least-squares analysis of the data.

number of 2 (36). DGPP did not affect the V_{max} value for the enzyme but did cause an increase in the K_m for PA (Fig. 7, inset). These results were consistent with DGPP being a competitive inhibitor with respect to PA. A K_i value for DGPP was calculated to be 0.35 mol %.

Effect of DGPP on PA Phosphatase Activity—PA phosphatase catalyzes the dephosphorylation of PA to yield diacylglycerol and P_i (37). We questioned whether DGPP could inhibit the activity of PA phosphatase purified (22) from *S. cerevisiae*. Activity was measured under the standard assay conditions determined for PA phosphatase (22). The PA concentration (3 mol %) used in these experiments was the surface concentration of PA near its K_m value (38). DGPP did not inhibit PA phosphatase activity (Fig. 8). On the contrary, the addition of DGPP to the assay system resulted in a dose-dependent stimulation of PA phosphatase activity (Fig. 8).³ We also examined whether PA phosphatase could catalyze the dephosphorylation of DGPP under the assay conditions for DGPP phosphatase and PA phosphatase. These experiments showed that PA phosphatase could not use DGPP as a substrate.

Identification of DGPP as the Reaction Product of PA Kinase in *S. cerevisiae*—PA kinase activity was measured in *S. cerevisiae* using the total membrane fraction as the source of enzyme under the assay conditions used for PA kinase activity from plants (5). The ³²P-labeled chloroform-soluble product of the reaction was analyzed by thin-layer chromatography. Autora-

³ Negatively charged phospholipids have been shown to stimulate the activity of pure PA phosphatase from *S. cerevisiae* (W.-I. Wu and G. M. Carman, manuscript in preparation).

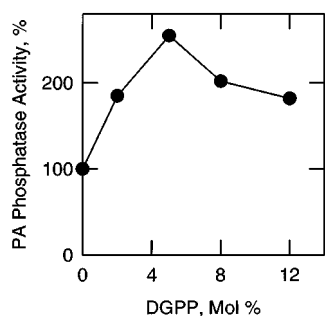


FIG. 8. **Effect of DGPP on PA phosphatase activity.** The activity of pure PA phosphatase was measured with 3 mol % [^{32}P]PA (molar concentration of 0.1 mM) in the presence of the indicated surface concentrations of DGPP.

diographic analysis of the thin-layer chromatography plate showed that the product of the reaction was indeed DGPP (Fig. 9). The amount of labeled DGPP on the chromatogram was determined by scintillation counting and used to calculate PA kinase activity. The specific activity of PA kinase was 7 pmol/min/mg. The heavily labeled spots near the bottom of the chromatogram shown in Fig. 9 were the products (polyphosphoinositides) of phosphoinositide kinases (1) present in yeast membranes (39).

Cellular Concentration of DGPP—Phospholipids were extracted from exponential phase cells and subjected to high performance liquid chromatography. A typical chromatogram of this analysis showed that DGPP was indeed a very minor phospholipid in *S. cerevisiae* (Fig. 10). DGPP eluted from the column with a retention time of 23.3 min and was well separated from the major phospholipids. Phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, and PA eluted from the column between 15–21 min (Fig. 10). The major phospholipids were not well separated from each other under the conditions used here to identify DGPP. DGPP accounted for 0.18 ± 0.02 mol % (4 determinations) of the major phospholipids in *S. cerevisiae*.

DISCUSSION

We undertook the purification of DGPP phosphatase from *S. cerevisiae* to facilitate well defined studies on the biochemical regulation of this novel enzyme of phospholipid metabolism. Purification of DGPP phosphatase required the solubilization of the enzyme from microsomal membranes with Triton X-100 followed by conventional chromatography steps performed in the presence of Triton X-100. The presence of Triton X-100 in the buffers used for enzyme purification was required to prevent aggregation of this integral membrane protein. The hydroxylapatite chromatography step resulted in two distinct peaks of DGPP phosphatase activity. Each peak of activity was purified separately by Mono Q chromatography. The seven-step purification procedure reported for the enzyme from hydroxylapatite peak I resulted in a DGPP phosphatase preparation that was essentially homogeneous as evidenced by native and SDS-polyacrylamide gel electrophoresis. DGPP phosphatase was identified in this preparation as a 34-kDa protein. Overall, DGPP phosphatase was purified 33,333-fold relative to the activity in the cell extract to a final specific activity of 150 $\mu\text{mol}/\text{min}/\text{mg}$. The enzyme from hydroxylapatite peak II was purified 24,666-fold over the activity in the cell extract to a final specific activity of 111 $\mu\text{mol}/\text{min}/\text{mg}$. This near homogeneous enzyme preparation also contained the 34-kDa protein, which was associated with DGPP phosphatase activity. The reason for the different chromatographic properties of the two DGPP phosphatase preparations is unclear. Additional studies are required to address these differences. The molecular mass

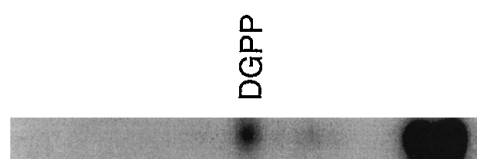


FIG. 9. **Identification of DGPP as the reaction product of PA kinase.** PA kinase activity was measured for 5 min with [γ - ^{32}P]ATP and PA as substrates using the total membrane fraction as the source of enzyme. Following the incubation, the chloroform-soluble ^{32}P -labeled product of the reaction was analyzed by thin-layer chromatography. The position of standard DGPP is indicated in the figure. The radiolabeled spots near the bottom (right of figure) of the chromatogram were polyphosphoinositides.

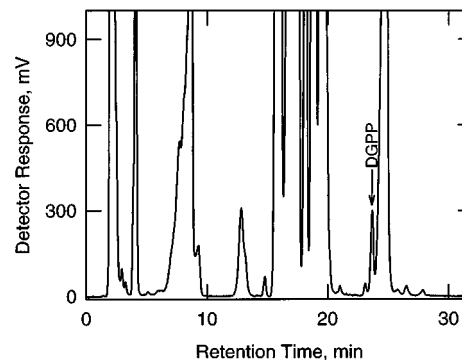


FIG. 10. **High performance liquid chromatography of DGPP.** Phospholipids were extracted from *S. cerevisiae* cells and analyzed by high performance liquid chromatography as described under "Experimental Procedures." The elution position (23.3 min) of DGPP is indicated in the figure. The elution positions of phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, and PA were between 15 and 21 min. The peaks in the chromatogram eluting before min 15 and after DGPP were not identified.

of native DGPP phosphatase in Triton X-100 micelles cannot be determined until the interaction of the enzyme with detergent has been quantitated (40).

The fold purifications and final specific activities of the DGPP phosphatase preparations described here were higher than any other phospholipid-dependent enzyme purified from *S. cerevisiae* (reviewed in Refs. 8 and 39). That such a high degree of purification was required to obtain purified DGPP phosphatase indicated that the abundance of this enzyme in *S. cerevisiae* was relatively low when compared with other phospholipid-dependent enzymes.

Pure DGPP phosphatase derived from hydroxylapatite peak I was used for the biochemical characterization of the enzyme. The enzyme had a broad pH optimum and required Triton X-100 for maximum activity. The enzyme did not have a divalent cation requirement, and activity was insensitive to Na_2EDTA . Divalent cations, especially Mn^{2+} ions, potentially inhibited DGPP phosphatase activity. DGPP phosphatase was also inhibited by NaF but was relatively insensitive to thioester-active compounds. Pyrophosphate and, to a lesser extent, ADP (which contains a pyrophosphate group) inhibited DGPP phosphatase activity. We did not test whether these compounds were substrates for DGPP phosphatase. Pyrophosphate is the specific substrate for inorganic pyrophosphatase in *S. cerevisiae* (41, 42). DGPP phosphatase differed from inorganic pyrophosphatase in that inorganic pyrophosphatase is a cytosolic enzyme, which is dependent on Mg^{2+} ions for activity (41, 42).

DGPP phosphatase catalyzed the dephosphorylation of the β phosphate of DGPP yielding PA and P_i . DGPP phosphatase activity was not inhibited by its product PA. This lack of product inhibition was consistent with the length of time in which the reaction was linear and the enzyme catalyzing the near

TABLE III
Kinetic constants for DGPP phosphatase and cellular concentrations of its substrates

Substrate or inhibitor	V_{\max}	K_m	V_{\max}/K_m	K_i	Cellular concentration
	units/mg	mol %		mol %	mol %
DGPP	172	0.55	313	0.35 ^a	0.18
PA	70	2.2	32	NI ^b	3

^a Inhibitor constant with respect to PA as substrate.

^b NI, not inhibitory.

quantitative conversion of DGPP to PA. Whereas the enzyme was not inhibited by PA, DGPP phosphatase catalyzed the dephosphorylation of PA. The dephosphorylation of PA was subsequent to the dephosphorylation of the β phosphate of DGPP. Moreover, the dephosphorylation of PA by DGPP phosphatase was potently inhibited by DGPP. DGPP was a competitive inhibitor with respect to PA and the K_i value for DGPP was similar to the K_m value for DGPP (Table III). Taken together, these results suggested that the DGPP and PA binding sites on the enzyme were the same.

DGPP phosphatase activity followed surface dilution kinetics (35) using Triton X-100/phospholipid-mixed micelles. The role of Triton X-100 in the assay of phospholipid-dependent enzymes is to form a mixed micelle with the phospholipid substrate and, therefore, provide a surface for catalysis (35). Indeed, DGPP phosphatase activity was dependent on the surface concentration of DGPP. The reaction of the dephosphorylation of PA also followed surface dilution kinetics. However, in contrast to the typical kinetic behavior (saturation kinetics) the enzyme exhibited toward DGPP, the dependence of activity on the surface concentration of PA was cooperative.

DGPP was clearly the preferred substrate for pure DGPP phosphatase based on the relative values for V_{\max} and K_m (Table III). Moreover, the specificity constant (V_{\max}/K_m) for DGPP was 10-fold higher than that for PA (Table III). Although the cellular concentration of DGPP was 16-fold lower than that of PA (Table III), an argument can be made for DGPP being the preferred substrate for the enzyme *in vivo*. PA, at a concentration 20-fold higher than that of DGPP, did not inhibit DGPP phosphatase activity, and DGPP potently inhibited the enzyme's ability to catalyze the dephosphorylation of PA. Moreover, the K_i value for DGPP was very close to its cellular concentration (Table III). Our studies, however, do not rule out the possibility that the enzyme could use PA as a substrate *in vivo*.

The enzyme in *S. cerevisiae* which is responsible for the dephosphorylation of PA is PA phosphatase (22). PA phosphatase has been purified and extensively characterized from *S. cerevisiae* (22, 33, 38, 43–45). DGPP phosphatase differed from PA phosphatase with respect to molecular mass, substrate specificity, cofactor requirement, and sensitivity to thioreactive agents. For example, PA phosphatase requires Mg^{2+} ions for activity and is inhibited by *N*-ethylmaleimide (22, 43).

If DGPP phosphatase was to play an important role in phospholipid metabolism, it was important for us to demonstrate that DGPP was synthesized in *S. cerevisiae*. Indeed, we demonstrated that *S. cerevisiae* possessed PA kinase activity and DGPP was identified in growing cells. PA kinase activity (7 pmol/min/mg) in *S. cerevisiae* was very low when compared with the activity (3.3 nmol/min/mg) in membranes from *C. roseus* (5). This low level of activity may be a reflection of a low level of PA kinase expression, the product's rapid hydrolyzation by DGPP phosphatase, and/or not knowing the correct assay conditions for the enzyme. Additional studies are needed to characterize PA kinase activity in *S. cerevisiae*. DGPP was previously not identified in *S. cerevisiae*. In previous studies

(reviewed in Ref. 39), a relatively large percentage of unidentified phospholipids have been labeled as "others." Perhaps DGPP was a minor phospholipid among the unidentified phospholipids described in previous studies. DGPP accounted for only 0.18 mol % of the major phospholipids in *S. cerevisiae*. The identification of DGPP was dependent on the procedure used to extract phospholipids. Method IIIB of Hanson and Lester (32) was the best method to extract DGPP. This method is commonly used for the extraction of polar lipids such as polyphosphoinositides and sphingolipids (32). This method also minimizes creation of artifacts such as lysolipids (32). The cellular concentration of DGPP was 3-fold lower than the K_m value (0.55 mol %) for DGPP. Thus, DGPP phosphatase activity would be expected to be very sensitive to changes in the cellular concentration of DGPP.

It is unclear what roles DGPP and DGPP phosphatase play in phospholipid metabolism. One could speculate that DGPP is the precursor of the PA used for phospholipid synthesis or neutral lipid synthesis, DGPP is the precursor of the PA which acts as a signaling molecule, and/or DGPP itself is a signaling molecule. The activity of DGPP phosphatase could regulate the levels of DGPP and PA in the cell. Since DGPP phosphatase also dephosphorylated PA, the enzyme may play a role in regulating diacylglycerol levels. The studies reported here provide the foundation for future molecular genetic studies directed toward understanding the roles DGPP and DGPP phosphatase play in phospholipid metabolism and cell growth.

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**Purification and Characterization of Diacylglycerol Pyrophosphate Phosphatase from
*Saccharomyces cerevisiae***

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