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Isolation and Characterization of the *Saccharomyces cerevisiae DPP1* Gene Encoding Diacylglycerol Pyrophosphate Phosphatase*

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Diacylglycerol pyrophosphate (DGPP) is involved in a putative novel lipid signaling pathway. DGPP phosphatase (DGPP phosphohydrolase) is a membrane-associated 34-kDa enzyme from *Saccharomyces cerevisiae* which catalyzes the dephosphorylation of DGPP to yield phosphatidate (PA) and then catalyzes the dephosphorylation of PA to yield diacylglycerol. Amino acid sequence information derived from DGPP phosphatase was used to identify and isolate the *DPP1* (diacylglycerol pyrophosphate phosphatase) gene encoding the enzyme. Multicopy plasmids containing the *DPP1* gene directed a 10-fold overexpression of DGPP phosphatase activity in *S. cerevisiae*. The heterologous expression of the *S. cerevisiae DPP1* gene in Sf-9 insect cells resulted in a 500-fold overexpression of DGPP phosphatase activity over that expressed in wild-type *S. cerevisiae*. DGPP phosphatase possesses a Mg^{2+}-independent PA phosphatase activity, and its expression correlated with the overexpression of DGPP phosphatase activity in *S. cerevisiae* and in insect cells. DGPP phosphatase was predicted to be an integral membrane protein with six transmembrane-spanning domains. The enzyme contains a novel phosphatase sequence motif found in a superfamily of phosphatases. A *dpp1*Δ mutant was constructed by deletion of the chromosomal copy of the *DPP1* gene. The *dpp1*Δ mutant was viable and did not exhibit any obvious growth defects. The mutant was devoid of DGPP phosphatase activity and accumulated (4-fold) DGPP. Analysis of the mutant showed that the *DPP1* gene was not responsible for all of the Mg^{2+}-independent PA phosphatase activity in *S. cerevisiae*.

Diacylglycerol pyrophosphate (DGPP)[1] is a novel phospholipid that contains a pyrophosphate group attached to diacylglycerol (DG) (Fig. 1) (1). DGPP has been found in a variety of plants (2, 3) and in the yeast *Saccharomyces cerevisiae* (4). This phospholipid is synthesized from phosphatidate (PA) and ATP via the reaction catalyzed by the membrane-associated enzyme PA kinase (1) and is dephosphorylated to PA via the reaction catalyzed by the membrane-associated enzyme DGPP phosphatase (Fig. 1) (4). The amounts of DGPP in wild-type *S. cerevisiae* and in plants are barely detectable (3, 4). For example, DGPP accounts for only 0.18 mol % of the major phospholipids in *S. cerevisiae* (4). The low abundance of DGPP is reminiscent of lipid signaling molecules such as the inositol-containing phospholipids (5–9). Recent studies indicate that the metabolism of DGPP is involved in a novel lipid signaling pathway. DGPP accumulates in plant tissues upon G protein activation through the stimulation of PA kinase activity (3), and metabolic labeling studies with *Catharanthus roseus* cells have shown that DGPP is metabolized rapidly to PA and then to DG (10). It has been suggested that DGPP may function as a signaling molecule (3, 4). Alternatively, the formation of DGPP may serve to attenuate the signaling functions of PA (11, 12).

DGPP phosphatase activity has been identified in *S. cerevisiae*, *C. roseus*, *Escherichia coli*, rat liver, pig liver, pig brain, and bovine brain (4, 10). The discovery of DGPP phosphatase in such a wide range of organisms suggests that it plays an important role in cell function. DGPP phosphatase has been purified to homogeneity from *S. cerevisiae* and has been characterized with respect to its enzymological and kinetic properties (4). The enzyme has a subunit molecular mass of 34 kDa (4). When DGPP is supplied as a substrate for the pure enzyme, it removes the β phosphate of DGPP to generate PA, and then removes the phosphate of PA to generate DG (Fig. 1) (4). Indeed, DGPP phosphatase can utilize PA as a substrate in the absence of DGPP, although the enzyme has a 10-fold higher specificity constant for DGPP (4). PA does not alter DGPP phosphatase activity (4). However, DGPP does competitively inhibit the PA phosphatase activity of the DGPP phosphatase enzyme (4). The DGPP phosphatase and PA phosphatase activities of the DGPP phosphatase enzyme are Mg^{2+}-independent and *N*-ethylmaleimide-insensitive (4). In addition, DGPP phosphatase activity is inhibited potently by Mn^{2+} ions (4). The PA phosphatase activity of the DGPP phosphatase enzyme is distinct from the conventional PA phosphatase enzymes (13–15) that are proposed to be used for the synthesis of phospholipids and triacylglycerols in *S. cerevisiae* (4). The conventional PA phosphatases (45-, 75-, and 104-kDa forms) have a Mg^{2+} ion requirement and are sensitive to inhibition by *N*-ethylmaleimide (13, 14, 16). The 45- and 104-kDa forms of the Mg^{2+}-dependent PA phosphatases do not utilize DGPP as a substrate (4). In fact, the 104-kDa PA phosphatase activity is stimulated by DGPP (4, 16).

DGPP phosphatase is an interesting enzyme insofar as the

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[1] The abbreviations used are: DGPP, diacylglycerol pyrophosphate; DG, diacylglycerol; PA, phosphatidate; PCR, polymerase chain reaction; kb, kilobase(s); bp, base pairs.

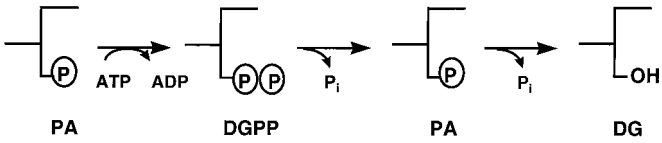

FIG. 1. **PA kinase, DGPP phosphatase, and PA phosphatase reactions.** The figure shows the structures of DGPP, PA, and DG and the PA kinase, DGPP phosphatase, and PA phosphatase reactions.

product of one reaction becomes the substrate for the subsequent reaction (Fig. 1). The regulation of this enzyme could control specific cellular pools of DGPP, PA, and DG and thus influence lipid signaling as well as overall lipid metabolism. The isolation of the gene encoding DGPP phosphatase is required for defined studies to examine the physiological roles of DGPP and DGPP phosphatase in eukaryotic cells. In this paper we report the isolation and initial characterization of the *DPP1* (diacylglycerol pyrophosphate phosphatase) gene encoding DGPP phosphatase in *S. cerevisiae*. This work represents the first report of the isolation of a eukaryotic gene encoding DGPP phosphatase. In addition, the chromosomal copy of the *DPP1* gene was deleted from *S. cerevisiae* to produce a *dpp1*Δ mutant. The initial characterization of the *dpp1*Δ mutant showed that the *DPP1* gene was not essential for cell growth and that *DPP1* was responsible for all of the detectable DGPP phosphatase activity in the cell.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were reagent grade. Growth medium supplies were purchased from Difco Laboratories. Protein assay reagent, molecular mass standards for SDS-polyacrylamide gel electrophoresis, and electrophoresis reagents were purchased from Bio-Rad. Polyvinylidene difluoride paper was purchased from Millipore. Restriction endonucleases, modifying enzymes, and recombinant Vent DNA polymerase with 5′- and 3′-exonuclease activity were purchased from New England Biolabs. Polymerase chain reaction (PCR) and sequencing primers were prepared commercially by Genosys Biotechnologies, Inc. The PCRScript™ AMP SK(+) cloning kit was from Stratagene, and the Yeastmaker™ yeast transformation system was obtained from CLONTECH. DNA sequencing kits were obtained from Applied Biosystems. The DNA size ladder used for agarose gel electrophoresis was purchased from Life Technologies, Inc. The baculovirus transfer vector pVL1392 was obtained from Invitrogen. Triton X-100 and bovine serum albumin were purchased from Sigma. Lipids were purchased from Avanti Polar Lipids and Sigma. Radiochemicals were purchased from NEN Life Science Products. Scintillation counting supplies and acrylamide for electrophoresis were from National Diagnostics. Silica Gel 60 thin-layer chromatography plates were from EM Science. *E. coli* DG kinase was obtained from Lipidex Inc.

Methods

Strains, Plasmids, and Growth Conditions—The strains and plasmids used in this work are listed in Tables I and II, respectively. Methods for yeast growth, sporulation, and tetrad analysis were performed as described previously (17, 18). Yeast cultures were grown in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or in complete synthetic medium minus inositol (19) containing 2% glucose at 30 °C. The appropriate amino acid of complete synthetic medium was omitted for selection purposes. *E. coli* strain DH5α was grown in LB medium (1% Tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) at 37 °C. Ampicillin (100 μg/ml) was added to cultures of DH5α-carrying plasmids. Media were supplemented with either 2% (yeast) or 1.5% (*E. coli*) agar for growth on plates. Yeast cell numbers in liquid media were determined by microscopic examination with a hemocytometer or spectrophotometrically at an absorbance of 600 nm. The inositol excretion phenotype (20) of yeast strains was examined on complete synthetic medium (minus inositol) by using growth of the inositol auxotrophic indicator strain MC13 (*ino1*) (19) as described by McGee *et al.* (21).

Amino Acid Composition and Amino Acid Sequence Analyses of DGPP Phosphatase—Pure DGPP phosphatase (160 pmol) was subjected to SDS-polyacrylamide gel electrophoresis (22) and transferred to polyvinylidene difluoride paper (23). A portion (11 pmol) of the sample was subjected to amino acid composition analysis (24). The remainder of the sample on polyvinylidene difluoride paper was digested with sequencing grade trypsin for 20 h at 37 °C as described by Aebersold *et al.* (25) using the buffer system of Fernandez *et al.* (26). The digested sample was washed twice with 0.06% trifluoroacetic acid. The digest was then subjected to narrowbore reverse phase high performance liquid chromatography (HPLC) using a Zorbax C-18 column (1 × 150 mm, inner diameter) as described by Lane *et al.* (24). Strategies for the selection of peptide fragments and their sequencing by automated Edman degradation were performed as described by Lane *et al.* (24). The amino acid composition and amino acid sequencing analyses were performed at the Harvard Microchemistry Facility (Cambridge, MA).

DNA Manipulations, Amplification of DNA by PCR, and DNA Sequencing—Plasmid and genomic DNA preparation, restriction enzyme digestion, and DNA ligations were performed by standard methods (18). Transformation of yeast (27, 28) and *E. coli* (18) were performed as described previously. Conditions for the amplification of DNA by PCR were optimized as described by Innis and Gelfand (29). The annealing temperature for the PCRs was 50 °C, and extension times were typically between 2.0 and 2.5 min at 72 °C. PCRs were run routinely for a total of 30 cycles. DNA sequencing reactions were performed with the Prism DyeDeoxy Terminator Cycle sequencing kit and analyzed with an automated DNA sequencer. Plasmid maintenance and amplifications were performed in *E. coli* strain DH5α. Amplification of the plasmid pDT1-DPP1 was performed in *E. coli* strain Epicurian Coli^{R}XL-1.

Isolation of the DPP1 Gene—We identified an open reading frame DNA sequence in the *Saccharomyces* Genome Data Base (locus YDR284C) whose predicted amino acid sequence matched exactly the amino acid sequences derived from the DGPP phosphatase protein. This gene was named *DPP1*. A 1.9-kb DNA fragment containing 600 bp of the putative *DPP1* promoter, its entire protein coding sequence, and 500 bp of the 3′-flanking sequence was obtained by PCR (primers: 5′-GTTACATTGTATCAGTCACAGGTACGG-3′ and 5′-GTCGACATTTATACATAGTATGTGTTAAGG-3′) using strain W303-1A genomic DNA as a template. The PCR product was ligated into the *Srf*I site of the pCRScript™ AMP SK(+) cloning vector resulting in the formation of pDT1-DPP1. This plasmid was digested with *Sal*I, which released a 2.0-kb fragment containing the open reading frame and approximately 600 bp of the promoter region and 500 bp of the 3′-untranslated region. This fragment was ligated into the *Sal*I site of YEp351, a multicopy *E. coli*/yeast shuttle vector containing the *LEU2* gene (30), to form plasmid pDT2-DPP1. This construct was then transformed into W303-1A (W303-1A/pDT2-DPP1) for the overexpression of the *DPP1* gene product.

A genomic copy of the *DPP1* gene was also isolated. The annotations accompanying the *DPP1* sequence from the data bases indicated that the *DPP1* gene flanked the 5′-end of the *ZIP1* gene, which was isolated by Sym *et al.* (31). We obtained the CEN plasmid p1219 that was used originally by Sym *et al.* (31) for isolation of the *ZIP1* gene. This plasmid contains an insert of yeast genomic DNA of approximately 20 kb. PCR and restriction enzyme analyses indicated that p1219 contained the *DPP1* open reading frame and its flanking sequences. A 2.1-kb insert, containing the *DPP1* gene, 740 bp of the promoter region, and 540 bp of the 3′-untranslated region, was released from p1219 by digestion with *Spe*I/*Ssp*I. This fragment was ligated into the *Spe*I/*Sma*I sites of pRS425, a multicopy *E. coli*/yeast shuttle vector containing the *LEU2* gene (32) to form plasmid pBZ1-DPP1. This construct was then transformed into W303-1A (W303-1A/pBZ1-DPP1) for the overexpression of the *DPP1* gene product.

*Construction of a dpp1*Δ *Mutant*—The plasmid pDT1-DPP1 was digested with *Sna*BI/*Bst*Z17I to remove the entire *DPP1* coding sequence. A 2.8-kb *TRP1*/Kan^{r} disruption cassette, derived from plasmid pJA52 (33) by *Sma*I digestion, was inserted into the blunt-ended *Sna*BI/*Bst*Z17I sites of plasmid pDT1-DPP1 to form the plasmid pDT3-dpp1Δ. A linear 3.5-kb *DPP1* deletion cassette was released from pDT3-dpp1Δ by digestion with *Sal*I. This DNA fragment was transformed into W303-1A to delete the chromosomal copy of the *DPP1* gene by the one-step gene replacement technique (34). Transformants were selected for their ability to grow on complete synthetic medium without tryptophan. Deletion of the chromosomal copy of the *DPP1* gene was confirmed by PCR (35) using the primers listed above with the extension time increased to 3.5 min. The template for the PCRs used to confirm the *DPP1* deletion was genomic DNA isolated from transformed colonies that grew on medium without tryptophan. One of the *dpp1*Δ mutants that we isolated was designated strain DTY1. DTY1 was transformed with pBZ1-DPP1 (DTY1/pBZ1-DPP1) to compliment the *dpp1*Δ mutant.

TABLE I
Strains used in this work

Strain	Relevant characteristics	Source or Ref.
E. coli		
DH5α	F⁻ ϕ80d*lac*ZΔM15 Δ(*lacZYA-arg*F)U169, *deo*R, *rec*A1, *end*A1 *hsd*R17(r_k^- m_k^+), *pho*A *sup*E44, λ⁻*thi*-1 *gyr*A96, *rel*A1	18
Epicurian Coli^R XL-1	Δ(*mcr*A)183Δ(*mcr*CB-*hsd*SMR-*mrr*)173, *endA1*, *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*, *lac*[*F' proAB*, *lacI^q*ZΔ, *M15*, Tn5(*Kan^r*)]	Stratagene
S. cerevisiae		
W303–1A	***MATa***, *leu2–3, 112, trp1–1, can1–100, ura3–1, ade2–1, his3–11,15*	57
W303–1B	*MATα, leu2–3, 112, trp1–1, can1–100, ura3–1, ade2–1, his3–11,15*	57
DTY1	*dpp1Δ::TRP1*/Kan^r derivative of W303–1A	This work
MC13	*MATα, ino1–13, lys2, can1*	19
OP1	*MATα, opi1–1, lys2*	20

TABLE II
Plasmids used in this work

Plasmid	Relevant characteristics	Source or Ref.
pCRScript™ AMP SK(+)	Cloning vector derived from the pBluescript II SK(+) phagemid, modified *Srf*I restriction endonuclease target sequence	Stratagene
pDT1-DPP1	*DPP1* gene derived from PCR ligated into the *Srf*I site of pCRScript™ AMP SK(+)	This work
YEp351	Multicopy *E. coli*/yeast shuttle vector containing *LEU2*	30
pDT2-DPP1	*DPP1* gene from pDT1-DPP1 ligated into the S*al*I site of YEp351	This work
p1219	CEN plasmid containing a genomic copy of *DPP1*	31
pRS425	Multicopy *E. coli*/yeast shuttle vector containing *LEU2*	32
pBZ1-DPP1	*DPP1* gene from p1219 ligated into the *Spe*I/*Sma*I sites of pRS425	This work
pJA52	Plasmid containing a *TRP1*/Kan^r disruption cassette	33
pDT3-dpp1Δ	*TRP1*/Kan^r disruption cassette from pJA52 ligated into the *Sna*BI/B*stZ*17I sites of plasmid pDT1-DPP1	This work
pDT4-DPP1	*DPP1* coding sequences from pBZ1-DPP1 ligated into the *Eco*RI/*Sal*I sites of pCRScript AMP SK(+)	This work
PVL1392	Baculovirus expression vector	Invitrogen
pWW1-DPP1	*DPP1* gene from pDT4-DPP1 ligated into the *Kpn*I/*Not*I site of PVL1392	This work

Recombinant Viral Expression of the S. cerevisiae DPP1 Gene in Insect Cells—Plasmid pBZ1-DPP1 was digested with *Mfe*I/*Sal*I to release the entire coding sequence of the *DPP1* gene. This DNA fragment was ligated into the *Eco*RI/*Sal*I sites of the pCRScript™ AMP SK(+) vector resulting in the formation of plasmid pDT4-DPP1. This plasmid was then digested with *Kpn*I/*Not*I, which released the *DPP1* open reading frame. The *DPP1* gene was then ligated into the *Kpn*I/*Not*I site of the baculovirus vector PVL1392 to form plasmid pWW1-DPP1. The pWW1-DPP1 plasmid was subsequently cotransfected with BaculoGold™ *Autographa californica* DNA (Pharmingen) into a monolayer of Sf-9 cells using the $CaCl_2$ method. The Sf-9 cells were routinely grown in TMNFH medium (36) containing 10% heat-inactivated fetal bovine serum. General procedures for the growth, maintenance, and infection of Sf-9 cells followed the methods described by O'Reilly *et al.* (36). Routine infection of Sf-9 cells for DGPP phosphatase expression used 1–2 × 10^7 cells grown in 75-cm^2 tissue culture flasks. The cells were infected at a viral multiplicity of 10 and grown in TMNFH medium with 10% heat-inactivated fetal bovine serum for 48 h. The infected cells were collected by gentle trituration with medium, harvested by centrifugation, and washed twice with phosphate-buffered saline. The final cell pellet was snap frozen over dry ice and stored at −80 °C. Cells that were not infected with virus, cells infected with virus containing the vector without the *DPP1* gene, and cells infected with recombinant virus containing the *S. cerevisiae* choline kinase gene (37) served as controls. The expression of the *S. cerevisiae* choline kinase gene in insect cells will be described elsewhere.

Preparation of Enzymes—DGPP phosphatase was purified to homogeneity from *S. cerevisiae* as described by Wu *et al.* (4). The protein used for amino acid sequencing was derived from Mono Q II (4). PA kinase was purified from suspension-cultured *C. roseus* cells as described by Wissing and Behrbohm (2). Yeast cell extracts (38) and total membranes (13) were prepared as described previously. Cell extracts derived from Sf-9 insect cells were prepared at 5 °C. Cells were washed in 50 mM Tris-HCl, pH 7.5, 0.3 M sucrose, 1 mM Na_2EDTA, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethanesulfonyl fluoride, 1 mM benzamide, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin and suspended (0.3 g, wet weight, of cells/ml) in the same buffer. Cells were disrupted by sonic oscillation for seven 30-s bursts, with a 1.5-min pause between bursts. The disrupted cell suspension was then centrifuged at 1,500 × *g* for 5 min to remove unbroken cells and cell debris.

Preparation of Substrates—DGPP standard and [β-^{32}P]DGPP were synthesized enzymatically using purified *C. roseus* PA kinase as described by Wu *et al.* (4). [^{32}P]PA was synthesized enzymatically from DG using *E. coli* DG kinase (39) as described previously (13).

Enzyme Assays—DGPP phosphatase activity was measured by following the release of water-soluble $^{32}P_i$ from chloroform-soluble [β-^{32}P]DGPP (5,000–10,000 cpm/nmol) as described by Wu *et al.* (4). The reaction mixture contained 50 mM citrate buffer, pH 5.0, 0.1 mM DGPP, 2 mM Triton X-100, 10 mM 2-mercaptoethanol, and enzyme protein in a total volume of 0.1 ml. DGPP phosphatase activity has been measured at pH 6.5 using Tris-maleate buffer (4). Reexamination of the pH optimum for the reaction showed that maximum activity was obtained at pH 5.0.[2] Mg^{2+}-independent PA phosphatase activity was measured by following the release of water-soluble $^{32}P_i$ from chloroform-soluble [^{32}P]PA (10,000 cpm/nmol) (40). The reaction mixture contained 50 mM Tris-maleate buffer, pH 6.5, 0.1 mM PA, 1 mM Triton X-100, 2 mM Na_2EDTA, 10 mM 2-mercaptoethanol, and enzyme in a total volume of 0.1 ml. All enzyme assays were conducted at 30 °C in triplicate. The average standard deviation of the assays was ± 5%. The enzyme reactions were linear with time and protein concentration. A unit of enzymatic activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product/min. Specific activity was defined as units/mg of protein. Protein concentration was determined by the method of Bradford (41) using bovine serum albumin as the standard.

Mass Analysis of DGPP—Phospholipids were extracted from *S. cerevisiae* cells using the solvent system consisting of 95% ethanol/water/diethyl ether/pyridine/ammonium hydroxide (15:15:5:1:0.018) as described by Hanson and Lester (42). Samples were dried *in vacuo*, dissolved in chloroform/methanol/water (15:15:5), and subjected to analytical normal phase HPLC as described by Wu *et al.* (4). The identity of DGPP was determined by comparing its elution profile with that of authentic DGPP (4). The cellular concentration of DGPP was calculated relative to the concentration of the major phospholipids in the extract (4).

RESULTS

Isolation of the S. cerevisiae DPP1 Gene and the Deduced Primary Structure of Its Encoded Protein—The amino acid sequence analysis of the pure DGPP phosphatase protein

[2] X. Chen and G. M. Carman, unpublished data.

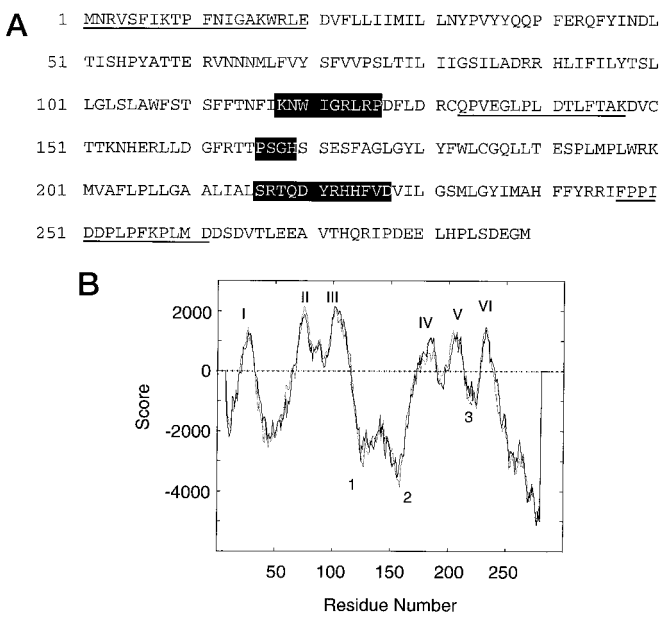

FIG. 2. **Deduced amino acid sequence of the *DPP1* gene and TMpred plot showing presumptive membrane-spanning regions.** *Panel A*, the *underlined* sequences correspond to the NH_2-terminal amino acid sequence and the two internal amino acid sequences derived from the pure DGPP phosphatase protein. The *shaded* amino acid residues in *black* and *boxed* areas indicate the three domains comprising the novel phosphatase sequence motif (44). Amino acids are numbered at the *left*. *Panel B*, the deduced amino acid sequence of the DGPP phosphatase protein was analyzed with the TMpred computer program (http://ulrec3.unil.ch:80/software) for presumptive transmembrane regions. The TMpred algorithm is based on the statistical analysis of a data base (TMbase) of naturally occurring transmembrane proteins (56). *Roman numerals* in the figure indicate presumptive membrane-spanning regions of the polypeptide chain. *Arabic numerals* in the figure indicate the regions that comprise the three domains of the novel phosphatase sequence motif.

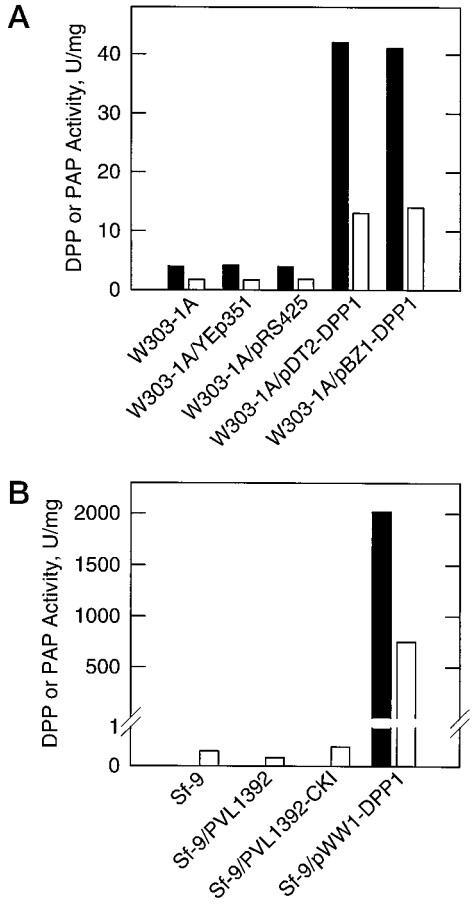

FIG. 3. **DGPP phosphatase and Mg^{2+}-independent PA phosphatase activities in *S. cerevisiae* strains and in Sf-9 insect cells overexpressing the *DPP1* gene.** Cell extracts were prepared from the indicated *S. cerevisiae* strains (*panel A*) and the indicated insect cells (*panel B*) and assayed for DGPP phosphatase (*DPP*, *solid bars*) and Mg^{2+}-independent PA phosphatase (*PAP*, *open bars*) activities as described under "Experimental Procedures."

yielded an NH_2-terminal amino acid sequence of MNRVSFIKTPFNIGAKWRLE and two internal amino acid sequences of QPVEGLPLDTLFTAK and FPPIDDPLPFKPLMD. These amino acid sequences aligned perfectly with the deduced amino acid sequence of an identified open reading frame DNA sequence in the *Saccharomyces* Genome Data Base (locus YDR284C) (Fig. 2*A*). In addition, the amino acid composition of the deduced protein matched the amino acid composition of pure DGPP phosphatase (data not shown). We named this gene *DPP1* for diacylglycerol pyrophosphate phosphatase. The *DPP1* gene is located on the right arm of chromosome IV (43). The *DPP1* gene coding sequence along with its 5′- and 3′-flanking sequences was isolated by PCR amplification using genomic DNA from strain W303-1A as the template. The *DPP1* gene and its flanking sequences were also isolated from plasmid p1219, a CEN-based plasmid that contains the *ZIP1* gene (31) on a 20-kb insert of genomic DNA. The PCR-derived and genomic-derived genes were sequenced twice by automated DNA sequence analysis. This analysis showed that both versions of the gene were identical and matched the sequence in the data base.

Inspection of the *DPP1* DNA sequence did not reveal any sequence motifs that would suggest the existence of introns in the gene. The predicted protein product is 289 amino acids in length, has a minimum subunit molecular mass of 33.5 kDa, and is predicted to be an integral membrane protein. During purification, the DGPP phosphatase enzyme is tightly associated with microsomal membranes, and the purified enzyme migrates as a 34-kDa protein after SDS-polyacrylamide gel electrophoresis analysis (4). This integral protein is predicted to have six transmembrane-spanning regions distributed over the entire polypeptide sequence (Fig. 2*B*). The deduced *DPP1* gene product contains a novel phosphatase sequence motif (44). This motif is comprised of three domains (44) that are localized to the hydrophilic surface of the membrane (Fig. 2*B*). The PSORT computer program (http://psort.nibb.ac.jp/form.html) predicts possible plasma membrane and Golgi body localization for the deduced amino acid sequence of the *DPP1* gene. The PROSITE Motif program (http://www.genome.ad.jp/sit/motif-.html) predicts that the *DPP1* gene product has three protein kinase C and two casein kinase II phosphorylation target sites.

DGPP Phosphatase and PA Phosphatase Activities in S. cerevisiae Cells and in Insect Cells Overexpressing the DPP1 Gene Product—The PCR-derived and the genomic-derived versions of the *DPP1* gene were used to construct multicopy plasmids for the overexpression of the *DPP1* gene product in *S. cerevisiae*. Cells bearing these multicopy plasmids were grown to the exponential phase of growth, and cell extracts were prepared and assayed for DGPP phosphatase activity. The plasmids containing both versions of the *DPP1* gene directed a 10-fold overexpression of DGPP phosphatase activity compared with cells not bearing a plasmid (Fig. 3*A*). Cells bearing multicopy plasmids without the *DPP1* gene also exhibited wild-type levels of DGPP phosphatase activity (Fig. 3*A*).

Pure DGPP phosphatase from *S. cerevisiae* possesses a Mg^{2+}-independent PA phosphatase activity (4). We questioned whether the overexpression of the *DPP1* gene product also directed the overexpression of PA phosphatase activity. Indeed,

the plasmids containing PCR-derived and the genomic-derived versions of the *DPP1* gene directed a 7–8-fold overexpression of PA phosphatase activity compared with cells not bearing a plasmid and with cells bearing plasmid without the *DPP1* gene (Fig. 3*A*). The specific activity of DGPP phosphatase was 3-fold higher than the Mg^{2+}-independent PA phosphatase activity in cells bearing the *DPP1* gene on multicopy plasmids (Fig. 3*A*).

In wild-type cells, DGPP phosphatase activity is associated with the membrane fraction of the cell (4). The cytosolic and total membrane fractions were isolated from cell extracts by differential centrifugation and used for the assay of DGPP activity. Almost all (92–95%) of the activity was associated with the total membrane fraction (data not shown).

The overexpression of DGPP phosphatase activity in yeast cells bearing the *DPP1* gene on multicopy plasmids supported the conclusion that the *DPP1* gene encoded the enzyme. To provide further support for this conclusion we used heterologous expression of the gene in Sf-9 insect cells. The *DPP1* gene was placed within the genome of baculovirus under control of the polyhedrin promoter and expressed by viral infection of Sf-9 cells. Fig. 3*B* shows the DGPP phosphatase activity found in Sf-9 cells with the *DPP1*-containing baculovirus compared with control cells. The specific activity of DGPP phosphatase in cell extracts from insect cells expressing the *DPP1* gene was 2,000 nmol/min/mg, whereas DGPP phosphatase activity was not detected in the extracts from the control insect cells. This level of DGPP phosphatase expression was equivalent to a 500-fold purification over that expressed in the cell extract of wild-type *S. cerevisiae* (Fig. 3*A*). Mg^{2+}-independent PA phosphatase activity was detected in the control Sf-9 insect cells. Infection of the cells with the baculovirus containing the *DPP1* gene also resulted in the massive overexpression of Mg^{2+}-independent PA phosphatase activity (Fig. 3*B*). The DGPP phosphatase activity was 2.7-fold greater than the Mg^{2+}-independent PA phosphatase activity in the insect cells infected with virus containing the yeast *DPP1* gene. These data provided strong evidence that the structural gene for DGPP phosphatase was *DPP1*.

Deletion of the DPP1 Gene and Initial Characterization of the dpp1Δ Mutant—The *DPP1* gene was deleted to examine whether the gene was essential for cell growth and to examine phenotypes that would shed light on the physiological role of DGPP phosphatase. The gene was deleted *in vitro* and introduced into the genome of haploid cells by homologous recombination as described under "Experimental Procedures." The strategy for the deletion of the chromosomal copy of the *DPP1* gene by replacement with the *TRP1*/Kan^r disruption cassette is shown in Fig. 4*A*. PCR amplification reactions using genomic DNA of mutant cells as a template confirmed that the *TRP1*/Kan^r disruption cassette had integrated at the *DPP1* locus of the chromosome (Fig. 4*B*). Haploid *dpp1*Δ mutant cells were viable and exhibited growth properties similar to wild-type control cells when grown vegetatively in complete synthetic medium and in YEPD medium at 30 °C. Microscopic examination of *dpp1*Δ mutant cells showed no apparent gross morphological differences compared with wild-type cells. Overall, these results indicated that the *DPP1* gene was not essential for cell growth under typical laboratory growth conditions.

If the *DPP1* gene encoded DGPP phosphatase, then the deletion of the *DPP1* gene should result in a loss in DGPP phosphatase activity as well as a loss of PA phosphatase activity. DGPP phosphatase activity was not detected in the *dpp1*Δ mutant (Fig. 5*A*). The level of detectability of the enzyme assay was 0.01 unit/mg. In contrast, the specific activity of the Mg^{2+}-independent PA phosphatase in the mutant was only 50% lower than the activity found in its wild-type parent (Fig. 5*A*).

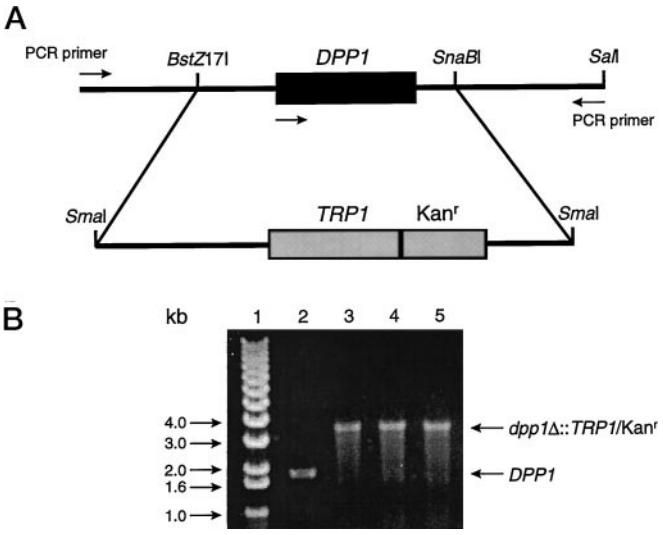

FIG. 4. **Physical map of the construct used for the deletion of the *DPP1* gene and confirmation of the deletion by PCR analysis.** *Panel A*, the figure shows the physical map of the construct used to delete the *DPP1* locus. Details of the deletion of the chromosomal copy of the *DPP1* gene by replacement with the *TRP1*/Kan^r disruption cassette are described under "Experimental Procedures." The *arrow* below the *wide bar* indicates the direction of transcription of the *DPP1* gene. *Panel B*, genomic DNA was prepared from strain W303-1A (*lane 2*) and from three independent isolates (*lanes 3–5*) containing a deletion of the *DPP1* allele and subjected to PCR amplification as described under "Experimental Procedures." The PCR products were subjected to agarose gel electrophoresis and stained with ethidium bromide. The positions of the *dpp1*Δ::*TRP1*/Kan^r allele and the intact *DPP1* allele are indicated in the figure. The positions of the DNA size standards (*lane 1*) are indicated in the figure.

Transformation of the *dpp1*Δ mutant with a multicopy plasmid containing the *DPP1* gene resulted in the overexpression of DGPP phosphatase and Mg^{2+}-independent PA phosphatase activities (Fig. 5*A*) to levels that were similar to that found in wild-type cells bearing the same multicopy plasmid (Fig. 3). Thus, the losses of DGPP phosphatase and Mg^{2+}-independent PA phosphatase activities in the *dpp1*Δ mutant were corrected by the introduction of the wild-type allele of the *DPP1* gene into the mutant.

We also examined what effect the deletion of the *DPP1* gene would have on the cellular concentration of DGPP, the substrate for the DGPP phosphatase reaction. The *dpp1*Δ mutant was grown to the exponential phase of growth, phospholipids were extracted, and the mass of DGPP was determined by HPLC analysis. As reported previously (4), DGPP accounted for less than 0.2 mol % of the total phospholipids found in cells containing the *DPP1* gene (Fig. 5*B*). Deletion of the *DPP1* gene resulted in a 4-fold increase in the cellular concentration of DGPP to 0.8 mol % of the total phospholipids (Fig. 5*B*). The overexpression of the *DPP1* gene in the *dpp1*Δ mutant resulted in a 27% decrease in the cellular concentration of DGPP compared with the wild-type parent.

The ability of the *dpp1*Δ mutant to mate and sporulate was examined. The *dpp1*Δ mutant was crossed with W303-1B to form a diploid that was heterozygous for the *DPP1* alleles. Diploid cells were induced to sporulate and analyzed for the segregation of the *TRP1* marker. Analysis of 21 tetrads showed a 2:2 segregation pattern for tryptophan prototrophy. Four randomly selected tryptophan prototrophs were examined for DGPP phosphatase activity. All of the tryptophan prototrophs were defective in DGPP phosphatase. The tryptophan auxotrophic progeny exhibited wild-type levels of DGPP phosphatase activity. Thus, mating and sporulation appear to be unaffected by the deletion of the *DPP1* gene.

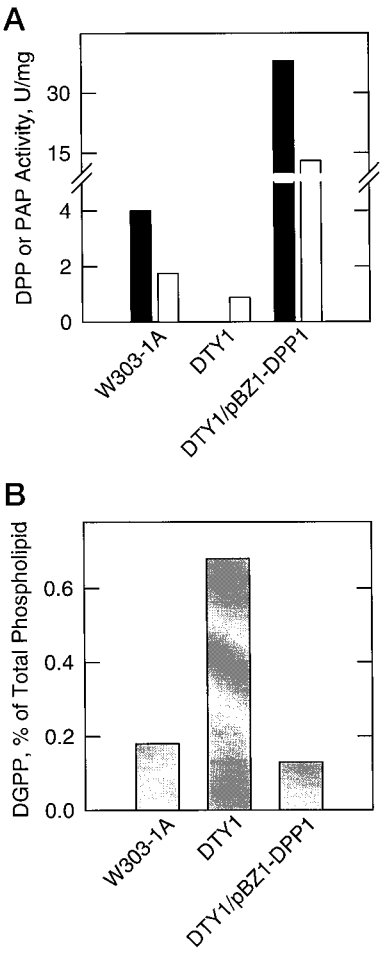

FIG. 5. **DGPP phosphatase and Mg^{2+}-independent PA phosphatase activities and the levels of DGPP in the *S. cerevisiae dpp1*Δ mutant.** *Panel A*, cell extracts were prepared from the indicated *S. cerevisiae* strains and assayed for DGPP phosphatase (*DPP*, *solid bars*) and Mg^{2+}-independent PA phosphatase (*PAP*, *open bars*) activities as described under "Experimental Procedures." *Panel B*, the amount of DGPP in the indicated *S. cerevisiae* strains was determined by HPLC analysis as described under "Experimental Procedures."

We have begun to characterize the physiology of cells that lack DGPP phosphatase activity. We examined the growth of the *dpp1*Δ mutant at different temperatures and on different carbon sources. Both *dpp1*Δ mutant cells and wild-type cells grew equally well at temperatures ranging from 16 °C to 37 °C when incubated on YEPD and on complete synthetic medium plates. There was no difference in the growth rate and maximum cell density of the *dpp1*Δ mutant compared with its parent when grown at 30 °C on medium with 2% glucose, 2% galactose, or 3% glycerol as the carbon source (data not shown).

A characteristic phenotype of mutants defective in the structural genes for several phospholipid biosynthetic enzymes (45, 46) is an inositol excretion phenotype (20). This phenotype is the result of the derepression of the *INO1* gene encoding inositol-1-phosphate synthase (45). The inositol excretion phenotype was examined for the *dpp1*Δ mutant using growth of the *ino1* mutant as an indicator of the phenotype. The *opi1* mutant, which excretes inositol (20) because of unregulated derepression of the *INO1* gene (45, 46), was used as a positive control. The *dpp1*Δ mutant did not exhibit an inositol excretion phenotype.

DISCUSSION

DGPP phosphatase is a novel enzyme recently purified and characterized from *S. cerevisiae* (4). The enzyme catalyzes the dephosphorylation of the β phosphate of DGPP to form PA and then catalyzes the dephosphorylation of the PA product to form DG (4). The substrates and products of the DGPP phosphatase reaction, namely DGPP, PA, and DG, have been shown to be involved in lipid signaling pathways (3, 11, 12, 47, 48). Thus DGPP phosphatase could play a major role in lipid signaling by regulating specific pools of these lipids. To gain insight into the function and regulation of DGPP phosphatase in eukaryotic cells we isolated the *DPP1* gene. The deduced amino acid sequence of the *DPP1* gene matched perfectly the amino acid sequences derived from pure DGPP phosphatase. Multicopy plasmids containing the *DPP1* gene directed the overexpression of DGPP phosphatase and Mg^{2+}-independent PA phosphatase activities in *S. cerevisiae*. Moreover, the heterologous expression of the *S. cerevisiae DPP1* gene in Sf-9 insect cells resulted in a massive overexpression of DGPP phosphatase and Mg^{2+}-independent PA phosphatase activities. The relative difference in the specific activities of DGPP phosphatase and Mg^{2+}-independent PA phosphatase from yeast cells and from insect cells overexpressing the *DPP1* gene was consistent with the relative difference in the V_{max} values determined for the pure DGPP phosphatase enzyme using DGPP and PA as substrates (4). Finally, the deletion of the *DPP1* gene in *S. cerevisiae* resulted in the loss of detectable DGPP phosphatase activity as well as a 4-fold accumulation in the cellular mass of DGPP. Collectively, these data provided a conclusive level of evidence for the identification of the *DPP1* gene as the structural gene encoding DGPP phosphatase in *S. cerevisiae*.

The loss of DGPP phosphatase activity in the *dpp1*Δ mutant does not rule out the possibility of another gene in *S. cerevisiae* which encodes a DGPP phosphatase activity. A second gene may exist whose product was not expressed under the growth conditions used in our experiments. Alternatively, a DGPP phosphatase may exist with assay requirements very different from those used in our experiments to measure DGPP phosphatase activity. For example, the deletion of the *S. cerevisiae PSD1* gene results in the loss of detectable phosphatidylserine decarboxylase activity (35, 49). Yet *S. cerevisiae* has a second gene (*PSD2*) that encodes a phosphatidylserine decarboxylase which is expressed at very low levels and has assay requirements different from the phosphatidylserine decarboxylase encoded by the *PSD1* gene (50, 51). The *dpp1*Δ mutant exhibited a 50% reduction in Mg^{2+}-independent PA phosphatase activity compared with the activity in wild-type cells. This indicated that the *DPP1* gene product was not responsible for all of the Mg^{2+}-independent PA phosphatase activity in the cell. We have isolated another gene from *S. cerevisiae* whose product exhibits Mg^{2+}-independent PA phosphatase activity. Its isolation and characterization will be the subject of a future paper.

The *pgpB* gene encodes DGPP phosphatase activity in *E. coli* (52). The *E. coli* DGPP phosphatase has been partially purified and characterized (52). The biochemical properties of the *E. coli* enzyme are similar to the pure DGPP phosphatase from *S. cerevisiae* (4) with respect to its substrate specificity for DGPP and for PA (52). In addition, the DGPP phosphatase and PA phosphatase activities exhibited by the *pgpB* gene product are Mg^{2+}-independent and *N*-ethylmaleimide-insensitive (52). The deduced protein products of the *E. coli pgpB* gene and the *S. cerevisiae DPP1* gene show regions of high homology which constitute the novel phosphatase sequence motif (44). Other than the phosphatase motif, these gene products show very little overall amino acid sequence homology (17% identity). The DGPP phosphatases from *S. cerevisiae* and *E. coli* are members of a superfamily of phosphatases that share amino acid sequence homology in the phosphatase sequence motif (44). This superfamily also includes the Mg^{2+}-independent and *N*-ethyl-

maleimide-insensitive form of PA phosphatase from mouse (53) and rat liver.[3] The rat liver Mg^{2+}-independent PA phosphatase can utilize DGPP as a substrate (54). However, this enzyme differs from the DGPP phosphatases from *S. cerevisiae* (4) and from *E. coli* (52) with respect to its substrate specificity for DGPP and for PA (54). Moreover, the mouse (53) and rat liver[3] Mg^{2+}-independent PA phosphatases share very little overall amino acid sequence homology with the DGPP phosphatases of *S. cerevisiae* (24 and 25% identity, respectively) and *E. coli* (19 and 20% identity, respectively).

Zhang *et al.* (55) have reported recently that Wunen, the product of the *wunen* gene in *Drosophila*, shows localized regions (that constitute the novel phosphatase sequence motif (44)) of high homology with the deduced protein products of the mouse Mg^{2+}-independent PA phosphatase cDNA (53) and an unidentified open reading frame DNA sequence in *S. cerevisiae*. We found here that the deduced yeast protein identified by Zhang *et al.* (55) is encoded by the *DPP1* gene. Wunen repels migrating germ cells during embryonic development (55). Based on the predicted amino acid sequence homologies of the *wunen* gene and of the mouse Mg^{2+}-independent PA phosphatase cDNA (53), Zhang *et al.* (55) have speculated that Wunen mediates its function through lipid signaling pathways involving Mg^{2+}-independent PA phosphatase activity. Wunen has not been examined for Mg^{2+}-independent PA phosphatase activity nor for DGPP phosphatase activity.

Construction of the *dpp1*Δ mutant showed that the *DPP1* gene was not essential for cell growth in *S. cerevisiae*. The vegetative growth and cell morphology of the *dpp1*Δ mutant were indistinguishable from those of its wild-type parent. The mutant could mate with a wild-type strain, and a *DPP1/dpp1*Δ heterozygote could sporulate. Although the *dpp1*Δ mutant lacked a dramatic phenotype, DGPP and DGPP phosphatase are ubiquitous in nature and have been shown in other systems possibly to play a role in cellular regulation (3, 10). The availability of the *DPP1* gene and the *dpp1*Δ mutant will permit a combination of genetic, molecular, and biochemical approaches to gain more a in-depth understanding of the roles DGPP phosphatase and DGPP play in lipid signaling and cell physiology.

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Isolation and Characterization of the *Saccharomyces cerevisiae DPP1* Gene Encoding Diacylglycerol Pyrophosphate Phosphatase

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