Mutagenesis of the Phosphatase Sequence Motif in Diacylglycerol Pyrophosphate Phosphatase from *Saccharomyces cerevisiae*†

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Received June 28, 1999

ABSTRACT: Diacylglycerol pyrophosphate (DGPP) phosphatase, encoded by the *DPP1* gene, is a membrane-associated enzyme in the yeast *Saccharomyces cerevisiae.* The enzyme removes the β phosphate from DGPP to form phosphatidate. The substrate and product of the DGPP phosphatase reaction play roles in lipid signaling and in cell metabolism. The deduced primary structure of the DGPP phosphatase protein contains a three-domain phosphatase sequence motif. In this work, we examined the hypothesis that the phosphatase sequence motif in the enzyme is involved in the DGPP phosphatase reaction. The amino acid residues Arg^{125}, His^{169}, and His^{223} in domains 1, 2, and 3, respectively, of the phosphatase sequence motif were changed to alanine residues by site-directed mutagenesis. The mutant $DPP1^{R125A}$, $DPP1^{H169A}$, and $DPP1^{H223A}$ alleles were cloned into a yeast shuttle vector and then expressed in a *dpp1*Δ *lpp1*Δ double mutant that lacks DGPP phosphatase activity. Northern blot and immunoblot analyses showed that the mutations in the phosphatase sequence motif did not affect the expression of the enzyme. The DGPP phosphatase activities of the R125A, the H169A, and the H223A mutant enzymes were 0.05, 9, and 0.03%, respectively, of the DGPP phosphatase activity of the wild-type enzyme. Enzymes with mutations in more than one domain of the phosphatase sequence motif had no measurable DGPP phosphatase activity. The R125A and H233A mutant DGPP phosphatase enzymes had reduced V_{max} and elevated K_m values for DGPP when compared with the wild-type enzyme. The H169A mutant enzyme had reduced V_{max} and K_m values when compared with the control. The specificity constants (V_{max}/K_m) for DGPP of the R125A mutant and H233A mutant enzymes were 4610-fold and 15 367-fold lower, respectively, when compared to the wild-type enzyme. The studies reported here indicated that the phosphatase sequence motif played an important role in the reaction catalyzed by the *S. cerevisiae* DGPP phosphatase.

Diacylglycerol pyrophosphate (DGPP)[1] phosphatase is a membrane-associated enzyme from *Saccharomyces cerevisiae* that catalyzes the removal of the β-phosphate from DGPP to form phosphatidate (PA), and then removes the phosphate from PA to form diacylglycerol (DAG) (*1*). The enzyme substrate DGPP is a novel phospholipid recently identified in *S. cerevisiae* (*1*). DGPP is synthesized from PA by the reaction catalyzed by PA kinase (*1*). Recent studies suggest that the metabolism of DGPP may be involved in a novel lipid-signaling pathway. DGPP is found in *S. cerevisiae* (*1*) and in plants (*2, 3*). The amounts of DGPP in these organisms are barely detectable (*1, 3*). The low abundance of this phospholipid is reminiscent of other lipid signaling molecules such as the inositol-containing phospholipids (*4*−*8*). DGPP accumulates in plant tissues upon G protein activation through the stimulation of PA kinase activity (*3*). Additional studies in *Catharanthus roseus* have shown that after DGPP is synthesized, it is rapidly metabolized to PA, and then to diacylglycerol (DAG) (*9*). The function of DGPP may be to attenuate the signaling functions associated with PA (*10, 11*) and/or that DGPP is the precursor of the PA that serves as a signaling molecule (*1, 3*). In addition, DGPP itself functions as a signaling molecule in mammalian cells (*12*). It potently activates mouse macrophages for enhanced secretion of arachidonic acid metabolites, an important event in the immunoinflammatory response of leukocytes (*12*). The DGPP phosphatase enzyme may function to regulate specific cellular pools of DGPP, PA, and DAG (*13*).

The *DPP1* (*14*) and *LPP1* (*15*) genes encode all of the DGPP phosphatase activity in *S. cerevisiae*. The *DPP1* and *LPP1* gene products also exhibit PA phosphatase activity (*14, 15*). The *DPP1*-encoded DGPP phosphatase is more abundant than the *LPP1*-encoded enzyme and it accounts for nearly all of the DGPP phosphatase activity in wild-type cells (*14, 15*). Biochemical analysis of *dpp1*Δ mutant, *lpp1*Δ mutant, and *dpp1*Δ *lpp1*Δ double mutant cells reveals that the *DPP1* and *LPP1* gene products regulate the cellular levels of DGPP and PA as well as phosphatidylinositol (*15*). In *S. cerevisiae*, phosphatidylinositol serves as the precursor for sphingolipids and the D-3 and D-4 phosphoinositides (*16, 17*). These molecules play prominent roles in lipid

† This work was supported in part by United States Public Health Service, National Institutes of Health Grant GM-28140.

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[1] The abbreviations used are: DGPP, diacylglycerol pyrophosphate; PA, phosphatidate; DAG, diacylglycerol; PCR, polymerase chain reaction.

10.1021/bi991472x CCC: $18.00
Published on Web 10/12/1999

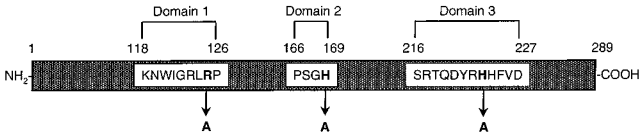

FIGURE 1: Phosphatase sequence motif of the *DPP1*-encoded DGPP phosphatase. The amino acid sequences that comprise the three domains of the phosphatase sequence motif within the DGPP phosphatase protein are indicated in the figure. The numbers on top of the diagram indicate the amino acid positions for each domain of the phosphatase sequence motif within the DGPP phosphatase protein. The conserved amino acid within each domain that was mutated to an alanine residue is indicated in the figure.

signaling in *S. cerevisiae* as well as in mammalian cells (*7*, *8*, *16*, *18*, *19*).

Studies to examine the reaction catalyzed by the DGPP phosphatase enzyme in a well-defined system are important to the understanding of the mode of action and control of this enzyme in vivo. Indeed, the *DPP1*-encoded enzyme has been purified to homogeneity and characterized with respect to its basic enzymological and kinetic properties (*1*). DGPP is the preferred substrate for the enzyme with a specificity constant 10-fold higher than that of PA (*1*). The DGPP phosphatase and PA phosphatase activities of the enzyme are Mg^{2+}-independent and *N*-ethylmaleimide-insensitive (*1*). In addition, the DGPP phosphatase activity of the enzyme is potently inhibited by Mn^{2+} ions (*1*) by a mechanism that involves the chelation of these ions to the pyrophosphate moiety of DGPP.[2]

A novel phosphatase sequence motif, which is shared by a superfamily of phosphatases, was first identified by Stukey and Carman (*20*) and then expanded further by Hemrika et al. (*21*) and Neuwald (*22*). The motif consists of three domains with the consensus sequences KXXXXXXRP (domain 1)−PSGH (domain 2)−SRXXXXXHXXXD (domain 3) where X is any amino acid (*20*). Amino acid segments ranging from 12 to 54 amino acids in length separate each of the three domains (*20*). The deduced primary structure of the yeast *DPP1*-encoded DGPP phosphatase (*14*) contains this novel motif (Figure 1) (*21, 22*). In this work, we examined the hypothesis that the conserved Arg^{125}, His^{169}, and His^{223} residues of the motif in the DGPP phosphatase would play an important role in enzyme activity. These amino acid residues were changed to alanine residues by site-directed mutagenesis. The R125A, H169A, and H233A mutant enzymes exhibited reduced DGPP phosphatase activity by a mechanism that affected the kinetic properties of the enzyme. This is the first report documenting that the phosphatase sequence motif plays a role in the reaction catalyzed by a lipid phosphate phosphatase.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were reagent grade. Growth medium supplies were purchased from Difco Laboratories. Restriction endonucleases, modifying enzymes, and recombinant Vent DNA polymerase with 5′ and 3′exonuclease activity were purchased from New England Biolabs. The DNA size ladder used for agarose gel electrophoresis was purchased from Life Technologies. Oligonucleotides were prepared commercially by Genosys Biotechnologies, Inc. The QuikChange site-directed mutagenesis kit was purchased from Stratagene. The Yeastmaker yeast transformation system was obtained from Clontech. The Prism DyeDeoxy DNA sequencing kit was obtained from Applied Biosystems. ProbeQuant G-50 columns were purchased from Amersham Pharmacia Biotech. Zeta Probe membranes, protein assay reagent, electrophoresis reagents, immunochemical reagents, and DEAE-Affi-Gel Blue were purchased from Bio-Rad. NEBlot kit was purchased from New England Biolabs. RNA size markers were purchased from Promega. Radiochemicals were purchased from NEN Life Science Products. Scintillation counting supplies and acrylamide solutions were from National Diagnostics. Bovine serum albumin, phenylmethylsulfonyl fluoride, benzamide, aprotinin, leupeptin, pepstatin, and nitrocellulose paper were purchased from Sigma. DGPP, PA, and DAG were obtained from Avanti Polar Lipids, Inc.

Strains, Plasmids, and Growth Conditions. The strains and plasmids used in this work are listed in Table 1. Methods for yeast growth were performed as described previously (*23*, *24*). Yeast cultures were grown in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or in complete synthetic medium minus inositol (*25*), 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 hemacytometer or spectrophotometrically at an absorbance of 600 nm.

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 (*24*). Transformation of yeast (*26*, *27*) and *E. coli* (*24*) were performed as described previously. Conditions for the amplification of DNA by PCR were optimized as recommended by the manufacturer's instructions of the site-directed mutagenesis kit. The annealing temperature for the PCR reactions was 55 °C with an extension time of 10 min at 68 °C. PCR reactions were routinely run for a total of 18 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.

Construction of Plasmids. The sequences of the oligonucleotide primers used for the construction of the *DPP1* mutant alleles are listed in Table 2. The $DPP1^{R125A}$, $DPP1^{H169A}$, and $DPP1^{H223A}$ mutations of domains 1, 2, and 3, respectively, of the phosphatase sequence motif were constructed by PCR using plasmid pDT1-DPP1 as the template (Table 1). The primers used for the mutations in Domains 1 (D1F and D1R), 2 (D2F and D2R), and 3 (D3F and D3R) incorporated *Kas*I, *Ban*I, and *Sac*I restriction sites, respectively, at the point of each mutation. These restriction sites were used to identify plasmids with the correct mutation. The $DPP1^{R125A,H169A}$ mutant was constructed with the primers for the H169A mutation using plasmid pDT5 ($DPP1^{R125A}$ mutant) as the template. The $DPP1^{R125A,H223A}$ mutant was

[2] G. M. Carman, unpublished data.

Table 1: Strains and Plasmids Used in This Work

strain or plasmid	relevant characteristics	source or ref
E. coli		
DH5α	F$^-$, ϕ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR, recA1, endA1, hsdR17*($r_k^-m_k^+$), *phoA, supE44*, λ^-*thi-1, gyrA96, relA1*	(*24*)
XL-1 Blue	*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac*[F' *proAB, lacl*^{q}*Z*ΔM15, *Tn10*(*Tet*^{r})]	Stratagene
S. cerevisiae		
TBY1	*MAT***a**, *leu2-3, 112, can1-100, ura3-1, ade2-1, dpp1*Δ::*TRP1*/Kan^r, *lpp1*Δ::*HIS3*/Kan^r	(*15*)
Plasmid		
pDT1-DPP1	*DPP1* gene ligated into the *Srf*I site of pCRScript AMP SK(+) used for mutagenesis	(*14*)
pDT5	*DPP1*^{R125A} derivative of pDT1-DPP1, mutation in domain 1	This work
pDT6	*DPP1*^{H169A} derivative of pDT1-DPP1, mutation in domain 2	This work
pDT7	*DPP1*^{H223A} derivative of pDT1-DPP1, mutation in domain 3	This work
pDT8	*DPP1*^{R125A,H169A} derivative of pDT1-DPP1, mutations in domains 1 and 2	This work
pDT9	*DPP1*^{R125A,H223A} derivative of pDT1-DPP1, mutations in domains 1 and 3	This work
pDT10	*DPP1*^{H169A,H223A} derivative of pDT1-DPP1, mutations in domains 2 and 3	This work
pDT11	*DPP1*^{R125A,H169A,H223A} derivative of pDT1-DPP1, mutations in domains 1, 2, and 3	This work
YEp352	Multicopy *E. coli*/yeast shuttle vector containing URA3	(*53*)
pDT12	*DPP1*^{R125A} gene from pDT5 ligated into the *Sal*I site of YEp352	This work
pDT13	*DPP1*^{H169A} gene from pDT6 ligated into the *Sal*I site of YEp352	This work
pDT14	*DPP1*^{H223A} gene from pDT7 ligated into the *Sal*I site of YEp352	This work
pDT15	*DPP1*^{R125A,H169A} gene from pDT8 ligated into the *Sal*I site of YEp352	This work
pDT16	*DPP1*^{R125A,H223A} gene from pDT9 ligated into the *Sal*I site of YEp352	This work
pDT17	*DPP1*^{H169A,H223A} gene from pDT10 ligated into the *Sal*I site of YEp352	This work
pDT18	*DPP1*^{R125,H169A,H223A} gene from pDT11 ligated into the *Sal*I site of YEp352	This work
pDT19	*DPP1* gene from pDT1-DPP1 ligated into the *Sal*I site of YEp352	This work

Table 2: Oligonucleotides Used in this Work

oligo	sequence	mutation
D1F[a]	5′-GAATTGGATTGGAAGACTGGCGCCAGATTTTCTAGATCG-3′	R125A
D1R[b]	5′-CGATCTAGAAAATCTGGCGCCAGTCTTCCAATCCAATTC-3′	
D2F	5′-GACAACTCCGTCAGGTGCCTCAAGTGAAAGCTTTGCAGG-3′	H169A
D2R	5′-CCTGCAAAGCTTTCACTTGAGGCACCTGACGGAGTTGTC-3′	
D3F	5′-GAACTCAAGATTACAGAGCTCATTTCGTCGATGTAATTTTAGG-3′	H233A
D3R	5′-CCTAAAATTACATCGACGAAATGAGCTCTGTAATCTTGAGTTC-3′	

[a] Forward orientation relative to sense strand. [b] Reverse orientation relative to sense strand.

constructed with the primers for the R125A mutation using plasmid pDT7 (*DPP1*^{H223A} mutant) as the template. The *DPP1*^{H169A,H223A} mutant was constructed with the primers for the H223A mutation using plasmid pDT6 (*DPP1*^{H169A} mutant) as the template. The *DPP1*^{R125A,H169A,H223A} mutant was constructed with the primers for the R125A mutation using plasmid pDT10 (*DPP1*^{H169A,H223A} mutant) as the template. The plasmid constructs bearing the mutated *DPP1* alleles were sequenced to verify the mutations.

Plasmids bearing the mutant and wild-type alleles of the *DPP1* gene were digested with *Sal*I to remove the *DPP1* gene. These DNA fragments were then ligated into the *Sal*I site of plasmid YEp352 to form the expression shuttle vectors pDT12−pDT19 (Table 1). Strain TBY1 (*dpp1*Δ *lpp1*Δ double mutant) was transformed with plasmids pDT12-pDT19 for the expression of the wild-type and mutant forms of the DGPP phosphatase enzymes.

RNA Isolation and Northern Blot Analysis. Total yeast RNA was isolated using the methods of Schmitt et al. (*28*) and Herrick et al. (*29*). Equal amounts (10 μg) of total RNA from each sample were resolved on a 1.1-% formaldehyde gel for 2.5 h at 100 V (*30*). The RNA samples were then transferred to Zeta Probe membrane by vacuum blotting. Prehybridization, hybridization with a specific probe, and washes to remove unbound probe were carried out according to manufacturer's instructions. The *DPP1* probe was a 0.87-kb fragment isolated from pDT1-DPP1 by *Mfe*I/*Bam*HI digestion. A 1-kb *Hind*III/*Kpn*I fragment of the phosphoglycerate kinase gene (*PGK1*) (*31*) was used as a constitutive standard and loading control. The *DPP1* and *PGK1* probes were labeled with [α-^{32}P]dATP using the NEBlot random primer labeling kit. Unincorporated nucleotides were removed using ProbeQuant G-50 columns. Images of radiolabeled species were acquired and quantified by PhosphorImaging analysis.

Preparation of Anti-DGPP Phosphatase Antibodies and Immunoblotting. The peptide sequence CTTKNHERLLDGFRTTPSG (residues 150−168 of the deduced protein sequence of *DPP1*) was synthesized and conjugated to carrier protein at Bio-Synthesis, Inc. (Lewisville, TX). This sequence is located between domains 1 and 2 of the phosphatase sequence motif (*14*). Antibodies were raised to the peptide in New Zealand White rabbits by standard procedures (*32*) at Bio-Synthesis, Inc. SDS-polyacrylamide gel electrophoresis (*33*) and immunoblotting (*34*) were performed as described previously. The density of the DGPP phosphatase bands on immunoblots was quantified by scanning densitometry. Immunoblot signals were in the linear range of detectability.

Preparation of Enzymes and Substrates. The total membrane fraction (*35*) and purified DGPP phosphatase (*1*) were prepared as described previously. [β-^{32}P]DGPP (*1*) and [^{32}P]-PA (*35, 36*) were synthesized enzymatically using *C. roseus* PA kinase and *Escherichia coli* DAG kinase, respectively.

Enzyme Assays, Protein Determination, and Analysis of Kinetic Data. DGPP phosphatase activity was measured by

following the release of water-soluble $^{32}P_i$ from chloroform-soluble [β-^{32}P]DGPP (10 000−20 000 cpm/nmol) (*1*). 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. The final membrane protein concentrations for the wild-type and mutant enzymes were typically 0.01 and 0.25 mg/mL, respectively. 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) (*37*). 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 enzyme reactions were linear with respect to 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 (*38*) using bovine serum albumin as the standard. Kinetic data were analyzed according to the Michaelis−Menten equation using the EZ-FIT Enzyme Kinetic Model Fitting Program (*39*).

RESULTS

Construction and Characterization of Mutants in the Phosphatase Sequence Motif of DGPP Phosphatase. Site-directed mutagenesis studies have shown that the conserved arginine residue in domain 1 and the conserved histidine residues in domains 2 and 3 that are found in of the phosphatase sequence motif of human glucose 6-phosphatase play essential roles in enzyme activity (*40*, *41*). On the basis of these data, we predicted that these conserved residues, which correspond to Arg^{125}, His^{169}, and His^{223} in the *DPP1*-encoded DGPP phosphatase, would be involved in the DGPP phosphatase reaction. The codons for Arg^{125}, His^{169}, and His^{223} were changed to alanine codons by site-directed mutagenesis. The mutations were made individually, and in combination for the enzyme. The mutant and wild-type *DPP1* alleles were expressed on a multicopy plasmid in a *dpp1*Δ *lpp1*Δ double mutant to obviate any effects due to the native DGPP phosphatase activities encoded by the *DPP1* and *LPP1* genes. Cells bearing multicopy plasmids containing the mutant alleles of the *DPP1* gene exhibited growth rates similar to cells bearing the wild-type allele when grown vegetatively at 30 °C. In addition, no morphological differences were observed in cells bearing the mutations in the *DPP1* gene. The fact that cells bearing the mutant enzymes did not exhibit any growth defects is not surprising since the *lpp1*Δ *dpp1*Δ double mutant itself shows no defects when compared with wild-type cells (*15*).

We examined whether the mutations in the phosphatase sequence motif affected the abundance of the mRNAs encoding DGPP phosphatase. Cells bearing the wild-type and mutant alleles were grown to the exponential phase of growth, and total RNA was extracted. The relative abundance of the mRNAs from these cells was determined by Northern blot analysis using a *DPP1* probe (Figure 2). The expression of *PGK1* mRNA was used as a loading control. PhosphorImaging analysis of the *DPP1* bands relative to the loading control indicated that there were essentially no differences in the *DPP1* mRNA levels between strains bearing the mutant and wild-type alleles of the *DPP1* gene.

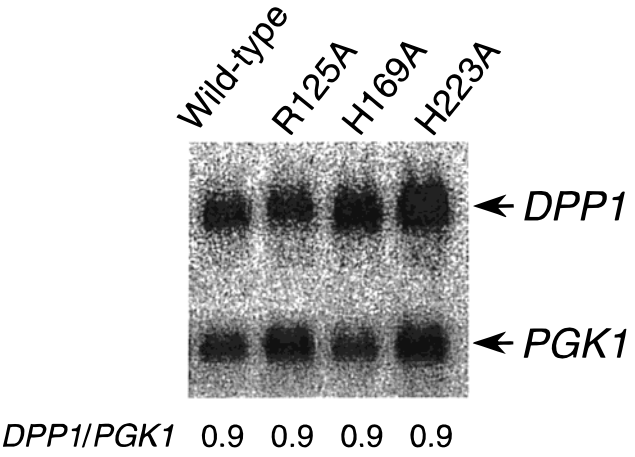

FIGURE 2: Effect of mutations in the phosphatase sequence motif on the abundance of DGPP phosphatase mRNA. Cells expressing the indicated *DPP1*-encoded wild-type and mutant DGPP phosphatase enzymes were grown in complete synthetic medium to the exponential phase of growth. Total RNA was extracted and the abundance of *DPP1* mRNA was determined by Northern blot analysis as described under Experimental Procedures. The amount of *DPP1* mRNA was normalized to that of *PGK1*, which was used as a loading control. The ratios of *DPP1*/*PGK1* mRNA for the wild-type and mutant enzymes are shown in the figure. The data shown is representative of two independent experiments.

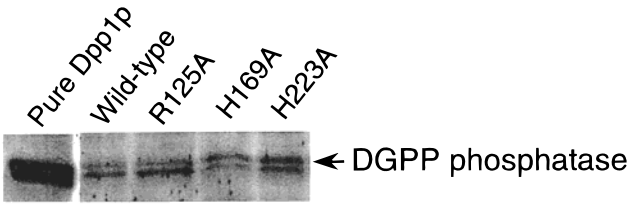

FIGURE 3: Immunoblot analysis of pure DGPP phosphatase and the effect of mutations in the phosphatase sequence motif on the levels of DGPP phosphatase. A 0.1 μg sample of pure DGPP phosphatase (Dpp1p) and 75 μg samples of membranes isolated from the cells expressing the indicated *DPP1*-encoded wild-type and mutant DGPP phosphatases were subjected to immunoblot analysis using a 1:250 dilution of anti-peptide antiserum as described under Experimental Procedures. A portion of the immunoblot is shown, and the position of the 34 kDa DGPP phosphatase enzyme is indicated. The data shown is representative of two independent experiments.

The expression of the wild-type and mutant DGPP phosphatase proteins was examined by immunoblot analysis. Antibodies were generated to a peptide sequence within the DGPP phosphatase protein, which recognized purified DGPP phosphatase (Figure 3). Immunoblot analysis using these antibodies showed that the wild-type and mutant forms of the enzyme were expressed in the membrane fraction of the *dpp1*Δ *lpp1*Δ double mutant transformed with the plasmids bearing the *DPP1* genes (Figure 3). Like the purified DGPP phosphatase (*1*), the wild-type and mutant forms of the enzyme migrated on SDS-polyacrylamide gels as a doublet with a subunit molecular mass of ∼34 kDa. Scanning densitometry of immunoblots of total membranes prepared from cells bearing plasmids with the wild-type and mutant alleles showed that there were essentially no differences in the DGPP phosphatase protein levels expressed. These data indicated that the mutations in the phosphatase sequence

Table 3: Effect of Mutations in the Phosphatase Sequence Motif on DGPP Phosphatase and PA Phosphatase Activities[a]

mutation	DGPP phosphatase		PA phosphatase	
	specific activity (nmol/min/mg)	relative activity (%)	specific activity (nmol/min/mg)	relative activity (%)
wild-type	290 ± 18	100	47.2 ± 1.5	100
vector[a]	0	0	0.13 ± 0.02	0.27
R125A	0.14 ± 0.02	0.05	0.14 ± 0.02	0.29
H169A	26.9 ± 0.6	9.27	0.23 ± 0.02	0.48
H223A	0.10 ± 0.03	0.03	0.13 ± 0.01	0.27
R125A,H169A	0	0	0.16 ± 0.02	0.33
R125A,H223A	0	0	0.15 ± 0.03	0.32
H169A,H223A	0	0	0.13 ± 0.02	0.27
R125A,H169A,H223A	0	0	0.14 ± 0.04	0.29

[a] Membranes isolated from cells expressing the indicated *DPP1*-encoded wild-type and mutant DGPP phosphatases on the multicopy plasmid were assayed for DGPP phosphatase and PA phosphatase activities as described under Experimental Procedures. [b] Membranes derived from *dpp1*Δ *lpp1*Δ mutant cells bearing the multicopy plasmid without the *DDP1* gene.

motif of DGPP phosphatase did not affect the expression of the enzyme or the targeting of the enzyme to the membrane.

Effect of Mutations in the Phosphatase Sequence Motif on DGPP Phosphatase and PA Phosphatase Activities. Total membranes were isolated from cells bearing the mutant and wild-type alleles of the *DPP1* gene and were used to measure DGPP phosphatase activity. To detect DGPP phosphatase activity of the mutant enzymes it was necessary to use membrane protein concentrations that were 25-fold greater than that used for the wild-type enzyme. The DGPP phosphatase assays of the mutant enzymes were linear with respect to protein concentration and with respect to the time of the assay. The DGPP phosphatase activities in the R125A mutant (domain 1) and H223A mutant (domain 2) enzymes were 0.05 and 0.03%, respectively, of the activity of the wild-type enzyme (Table 3). The effect of the mutation in domain 2 of the motif was less detrimental to DGPP phosphatase activity when compared with the mutations in domains 1 and 3. The DGPP phosphatase activity of the H169A mutant enzyme was 9% of the activity of the control enzyme (Table 3). We also examined the effects of mutations in multiple domains of the phosphatase sequence motif on DGPP phosphatase activity. Mutations in more than one of the domains of the motif resulted in a total loss of measurable DGPP phosphatase activity (Table 3). The expression of the DGPP phosphatase enzyme in the multiple domain mutants was confirmed by immunoblot analysis (data not shown).

The *DPP1*-encoded DGPP phosphatase of *S. cerevisiae* exhibits a Mg^{2+}-independent PA phosphatase activity (*1, 14*). We questioned whether the mutations in the phosphatase sequence motif would have an effect on this activity (Table 3). Indeed, the mutations in domains 1 and 3, and the multiple domain mutations caused reductions in PA phosphatase activity near the basal level of activity observed in membranes derived from cells not expressing the *DPP1* gene (Table 3). The *dpp1*Δ *lpp1*Δ double mutant possesses a small, but measurable amount of Mg^{2+}-independent PA phosphatase activity that is not encoded by the *DPP1* and *LPP1* genes (*15*). The PA phosphatase activity in the membranes derived from cells bearing the mutation in domain 2 was 1.8-fold greater than the basal activity of membranes from cells not expressing the *DPP1* gene (Table 3). This level of PA phosphatase activity (above the basal level) was 0.2% of the activity expressed in the wild-type control membranes.

Effect of Mutations in the Phosphatase Sequence Motif on the Dependence of DGPP Phosphatase Activity on the Surface Concentration of DGPP. The wild-type DGPP phosphatase enzyme follows surface dilution kinetics (*1*). Thus, the kinetic analysis of the mutant DGPP phosphatases was performed using Triton X-100/DGPP-mixed micelles. The detergent/phospholipid micelle system permits the kinetic analysis of lipid-dependent enzymes in an environment that mimics the surface of a membrane (*42*). Accordingly, the concentration of DGPP in the mixed micelles was expressed as a surface concentration in mol % as opposed to a molar concentration (*42*). As described previously for the purified DGPP phosphatase (*1*), the wild-type enzyme displayed Michaelis−Menten kinetics with respect to the surface concentration of DGPP (Figure 4A). The V_{max} for the reaction was 318 units/mg, and the K_m value for DGPP was 0.23 mol %. This K_m value was slightly lower than the value determined previously for the purified enzyme (*1*). This difference may be due to the fact that the enzyme used here was associated with membranes, which contain the enzyme's substrate DGPP (*1*). Nevertheless, the kinetic constants determined for the membrane-associated wild-type enzyme were used for comparison to the constants determined for the mutant membrane-associated enzymes. The R125A, H169A, and H233A mutations in the DGPP phosphatase resulted in decreases in V_{max} values of 99.9 (Figure 4B), 90 (Figure 4C), and 99.9% (Figure 4D), respectively, when compared with the wild-type control enzyme (Figure 4A). Note the differences in the *Y*-axis labels in Figure 3. The mutations in domains 1 (Figure 4B) and 3 (Figure 4D) caused increases in the K_m values for DGPP of 2.7- and 4.7-fold, respectively, when compared with the control enzyme. The mutation in domain 2 resulted in an 11.5-fold decrease in the K_m value for DGPP (Figure 4C). Note the difference in the *X*-axis label of panel C in Figure 4. The specificity constants for DGPP of the R125A mutant and H233A mutant enzymes were 4,610- and 15,367-fold lower, respectively, when compared to the specificity constant of the wild-type control enzyme (Table 4). On the other hand, the specificity constant for DGPP of the H169A mutant enzyme was not significantly different from that of the wild-type enzyme (Table 4).

Effect of Mutations in the Phosphatase Sequence Motif on the Temperature Stability of DGPP Phosphatase. We questioned whether the mutations in the phosphatase se-

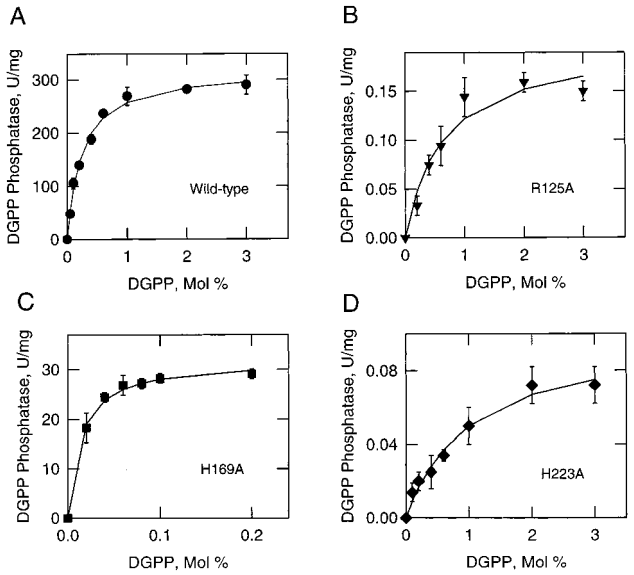

FIGURE 4: Effect of mutations in the phosphatase sequence motif on the dependence of DGPP phosphatase activity on the surface concentration of DGPP. Membranes isolated from cells expressing the *DPP1*-encoded wild-type (panel A), R125A mutant (panel B), H169A mutant (panel C), and H223A mutant (panel D) DGPP phosphatases were assayed for DGPP phosphatase activity as a function of the surface concentration (mol %) of [β-^{32}P]DGPP as described under Experimental Procedures. The molar concentration of DGPP was held constant at 0.1 mM while the Triton X-100 concentration was varied. The values reported were the average of three separate experiments ± SD. The curves drawn were the result of the analysis of the data according to the Michaelis−Menten equation.

Table 4: Effect of Mutations in the Phosphatase Sequence Motif on the Kinetic Constants of DGPP Phosphatase

mutant	V_{max}(app) (nmol/min/mg)	K_m(app) (mol %)	V_{max}/K_m (mol %$^{-1}$ nmol min^{-1} mg^{-1})
wild-type	318 ± 20	0.23 ± 0.02	1383
R125A	0.19 ± 0.04	0.63 ± 0.03	0.30
H169A	32 ± 1	0.02 ± 0.001	1600
H223A	0.10 ± 0.03	1.1 ± 0.05	0.09

quence motif of DGPP phosphatase would have an effect on the temperature stability of the enzyme. Membranes of the wild-type and mutant enzymes were incubated for 10 min at temperatures ranging from 30 to 60 °C. After incubation, the enzymes were cooled in an ice bath for 10 min to allow for protein renaturation followed by the measurement of DGPP phosphatase activity at 30 °C. The wild-type and mutant DGPP phosphatase enzymes were thermally stable to temperatures up to 40 °C (Figure 5). Whereas the wild-type and H169A mutant enzymes were stable to 60 °C, the R125A mutant and H223A mutant enzymes were thermally labile at temperatures above 40 °C (Figure 5).

DISCUSSION

The phosphatase sequence motif in the *DPP1*-encoded DGPP phosphatase is conserved in a superfamily of phosphatase enzymes (*20*). These phosphatases include DGPP phosphatases (*14, 43, 44*), Mg^{2+}-independent PA phosphatases (*15, 45*), sphingoid base phosphate phosphatase (*43, 46*), acid phosphatase (*47*), glucose 6-phosphatase (*41*), and

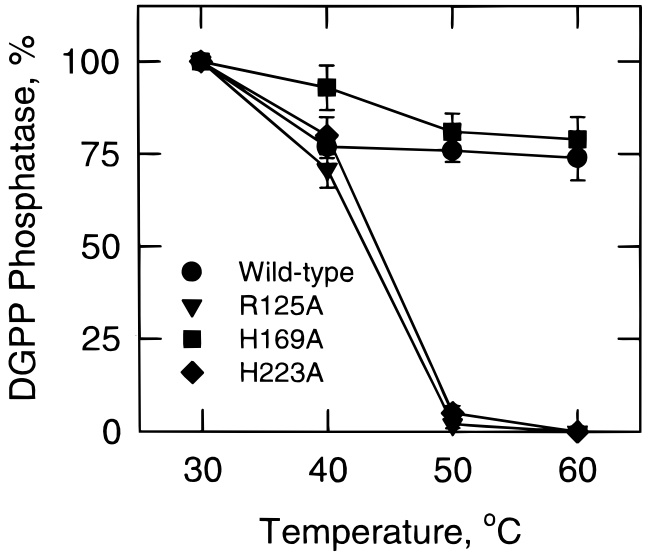

FIGURE 5: Effect of mutations in the phosphatase sequence motif on the temperature stability of DGPP phosphatase. Membranes isolated from cells expressing the indicated *DPP1*-encoded wild-type and mutant DGPP phosphatase enzymes were incubated at the indicated temperatures for 10 min. After the incubations the samples were cooled on ice for 10 min followed by the measurement of DGPP phosphatase activity at 30 °C as described under Experimental Procedures. The values reported were the average of three separate experiments ± SD.

vanadium-containing chloroperoxidase (*48*). Data derived from crystal structure analysis of chloroperoxidase and mutational studies with glucose 6-phosphatase have indicated that the conserved arginine residue in domain 1 and the conserved histidine residues in domains 2 and 3 are involved in the coordination and hydrolysis of a phosphate ester (reviewed in ref *20*). On the basis of this information, we predicted that Arg^{125}, His^{169}, and His^{223} in domains 1, 2, and 3, respectively, of the motif in the *DPP1*-encoded DGPP phosphatase (Figure 1), play an important role in the reaction of the enzyme.

The DGPP phosphatase activities of the R125A, H169A, and H223A mutant enzymes were 0.05, 9, and 0.03%, respectively, of the activity exhibited by the wild-type enzyme. The decreased phosphatase activities of the mutant enzymes were not due to defects in enzyme expression or proper targeting to the membrane. Northern blot and immunoblot analyses showed that the expression of the mutant DGPP phosphatases in *dpp1*Δ *lpp1*Δ cells were expressed in membranes essentially the same as that of the wild-type enzyme. Owing to the membrane-associated nature of the DGPP phosphatase, we were unable to examine structural properties of the wild-type and mutant enzymes. Multiple domain mutations resulted in a total loss of measurable DGPP phosphatase activity. This suggested that more than one domain of the motif be involved in substrate positioning and/or catalysis. This is in agreement with the crystal structure of chloroperoxidase, which shows that all three domains of the motif are in close proximity (*49*). The effects of the R125A and H233A mutations on the PA phosphatase activity of the enzyme were similar to the effects of these mutations on the DGPP phosphatase activity of the enzyme. However, the H169A mutation reduced the DGPP phosphatase activity to 9% of the control activity whereas this mutation reduced the PA phosphatase activity to about 0.2%

of the control activity. Substrate dependence and inhibitor studies indicate that the DGPP and PA binding sites in the DGPP phosphatase are the same (*1*). However, DGPP phosphatase has a specificity constant for DGPP that is 10-fold greater than that for PA (*1*). In addition, the DGPP phosphatase activity of the enzyme exhibits Michaelis−Menten kinetics toward DGPP, whereas the PA phosphatase activity exhibits positive cooperative kinetics toward PA (*1*). Thus, the differences in the effects of the H169A mutation on the DGPP phosphatase and PA phosphatase activities may be a reflection of the different structures (i.e., pyrophosphate moiety vs a phosphate moiety) of the two substrates that bind to the active site of the enzyme.

A kinetic analysis was performed to examine the mechanism of the loss of DGPP phosphatase activity in the phosphatase sequence motif mutants. The mutations in domains 1 and 3 caused a loss of DGPP phosphatase activity by a mechanism that involved an apparent decrease in substrate binding or positioning (as reflected in the increases in K_m values) and a decrease in catalysis (as reflected in the decrease in V_{max} values). Alterations in kinetic constants have been reported for mutations in the CDP-alcohol phosphotransferase motif of the cholinephosphotransferase of *S. cerevisiae* (*50*). The loss of DGPP phosphatase activity due to the mutation in domain 2 involved a decrease in catalysis. The H169A mutation caused an apparent increase in substrate binding or positioning (as reflected in an 11.5-fold decrease in the K_m value for DGPP).

The wild-type DGPP phosphatase enzyme was thermally stable to 60 °C. The chloroperoxidase enzyme, which contains the phosphatase sequence motif (*48*), also exhibits a relatively high degree of thermostability (*51*). The stability of enzymes to thermal denaturation is controlled by a number of factors, including disulfide bridges and hydrophobic and electrostatic interactions (*52*). The thermostability of the chloroperoxidase is attributed to compact packing of helices within the structure of the protein affording a stabilizing hydrophobic effect (*49*). The association of the wild-type enzyme with the membrane and/or its substrate DGPP within the membrane surface may have contributed to its thermostability. The three DGPP phosphatase mutant enzymes were stable to 40 °C, however, only the H169A mutant enzyme was stable to 60 °C. In fact, the H169A mutant enzyme was slightly more stable to thermal denaturation when compared with the wild-type enzyme. The thermostability of the wild-type and mutant enzymes correlated with the K_m values determined for the substrate DGPP. The wild-type and H169A DGPP phosphatases had relatively low K_m values, whereas the R125A and H223A enzymes had relatively high K_m values (Table 4). The K_m values for the R125A and H223A mutants were 3.5- and 6-fold greater, respectively, than the concentration of DGPP (0.18 mol %) in *S. cerevisiae* membranes (*1*). Thus, the apparent decreases in the affinities of the R125A and H223A mutants for DGPP may have contributed to the lability of these enzymes above 40 °C. These explanations maybe an oversimplification of the mechanism involved in the thermostability of the enzyme and alternative explanations can be made. Additional studies will be required to address this issue, but must await defined structure−function studies that utilize purified enzyme preparations. Nevertheless, the temperature stability of all the DGPP phosphatase enzymes at the assay temperature of 30 °C was similar. Thus, temperature would not be expected to have an impact on the activities of the mutant enzymes.

In summary, the data reported here supported the conclusion that the Arg^{125}, His^{169}, and His^{223} of the phosphatase sequence motif in DGPP phosphatase played important roles in the activity of the enzyme. This work provides the foundation for future structure−function studies on the yeast DGPP phosphatase and will facilitate studies on a variety of lipid phosphate phosphatase enzymes in lipid metabolism.

ACKNOWLEDGMENT

We thank Darin B. Ostrander for sequencing the mutant *DPP1* genes and thank Richard Ludescher for helpful discussions.

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BI991472X