The Saccharomyces cerevisiae PHM8 Gene Encodes a Soluble Magnesium-dependent Lyosphosphatidic Acid Phosphatase*<sup>S</sup>

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Venky Sreedhar Reddy<sup>1</sup>, Arjun Kumar Singh, and Ram Rajasekharan<sup>2</sup>

From the Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India

Phosphate is the essential macronutrient required for the growth of all organisms. In Saccharomyces cerevisiae, phosphatases are up-regulated, and the level of lysophosphatidic acid (LPA) is drastically decreased under phosphate-starved conditions. The reduction in the LPA level is attributed to PHM8, a gene of unknown function. phm8Δ yeast showed a decreased LPA-hydrolyzing activity under phosphate-limiting conditions. Overexpression of PHM8 in yeast resulted in an increase in the LPA phosphatase activity in vivo. In vitro assays of the purified recombinant Phm8p revealed magnesium-dependent LPA phosphatase activity, with maximal activity at pH 6.5. The purified Phm8p did not hydrolyze any lipid phosphates other than LPA. In silico analysis suggest that Phm8p is a soluble protein with no transmembrane domain. Site-directed mutational studies revealed that aspartate residues in a DXXDXT motif are important for the catalysis. These findings indicated that LPA plays a direct role in phosphate starvation. This is the first report of the identification and characterization of magnesium-dependent soluble LPA phosphatase.

Phosphate is an essential macronutrient required for the growth of all organisms. It is indispensable for the synthesis of nucleic acids, phospholipids, and high energy molecules, such as ATP and GTP. Therefore, it is necessary for the organisms to have evolved ways of sensing extracellular phosphate, scavenging it from the environment, and transporting it into the cell under phosphate-deprived conditions. Such activities are carried out by the gene products that are up-regulated during the phosphate starvation (1).

Phosphate is the most common functional group present in the metabolome. Under phosphate-limiting conditions, the phospholipids may act as phosphate source. In Arabidopsis thaliana, the amount of membrane phospholipids is decreased due to the up-regulation of a phosphatidylcholine (PC)-specific phospholipase C, and a nonphosphorous digalactosyldiacylglycerol level is increased under phosphate-limiting conditions (2, 3). In Monodus subterraneus, an increase in accumulation of triacylglycerol and a concomitant decrease in the level of phospholipids was observed (4). Under similar conditions, a photosynthetic bacterium Rhodobacter sphaeroides accumulated betaine lipid, diacylglycerol N-trimethylhomoserine, and sulfolipid, sulfoquinovosyldiacylglycerol by replacing PC and phosphatidylglycerol (5, 6).

In Saccharomyces cerevisiae, it was observed that ~550 genes are up-regulated under phosphate-deprived conditions (7). Among these, phosphatases represent a small fraction. Understanding the role of these enzymes during phosphate starvation is important because of the direct involvement of phosphatases in phosphate acquisition. The present study was aimed at deciphering the role of unknown phosphatase(s) in phospholipid metabolism under phosphate-deprived conditions. We have found that the level of lysophosphatidic acid (LPA) was reduced under phosphate-deprived conditions in S. cerevisiae. It has been shown earlier that LPA is the important intermediate for the biosynthesis of phospholipids and neutral lipids (8, 9). The decrease in LPA concentration in our study is attributed to PHM8 (phosphate metabolism gene 8), which is induced during phosphate starvation. Phm8p has a phosphatase motif within the haloacid dehalogenase (HAD)-like domain. The HAD superfamily includes enzymes that catalyze carbon or phosphoryl group transfer reaction on a diverse range of substrates. The majority of enzymes in this superfamily are involved in phosphate transfer (10, 11). In vivo labeling of Phm8p-expressing Escherichia coli and yeast cells and in vitro assays with the purified recombinant Phm8p suggest that the PHM8 encodes a soluble protein that dephosphorylates LPA in a magnesium-dependent manner. However, magnesium-independent soluble LPA phosphatases were reported from Arachis hypogaea (12) and bovine brain (13). In addition, the membrane-bound, magnesium-independent lipid phosphatases LPP1 (14) and DPP1 (15) are shown to hydrolyze PA, diacylglycerol pyrophosphate, and LPA. Here, we report the identification of the magnesium-dependent soluble LPA phosphatase that is up-regulated under phosphate-limiting conditions.

EXPERIMENTAL PROCEDURES

Materials—S. cerevisiae (BY4741) and PHM8 knock-out (phmΔ mutant) in BY4741 strains were obtained from Euroscarf (Frankfurt, Germany). [32P]Orthophosphate (3000 sulfonic acid; ESI-MS, electrospray ionization-mass spectrometry; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

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<sup>S</sup> The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

1. Recipient of a Council of Scientific and Industrial Research fellowship, Government of India, New Delhi.
2. To whom correspondence should be addressed: Lipid Laboratory, Dep. of Biochemistry, Indian Institute of Science, Bangalore 560012, India. Tel: 91-80-23602627; Fax: 91-80-23600814; E-mail: lipid@biochem.iisc.ernet.in.
3. The abbreviations used are: PC, phosphatidylcholine; LPA, lysophosphatic acid; SM, synthetic medium; HAD, haloacid dehalogenase; PA, phosphatidic acid; NTA, nitrilotriacetic acid; Mes, 4-morpholinolmethane-

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acetic acid (Ni$^{2+}$/H$_{11002}$ (SM prepared as described earlier (16). Phosphate-free medium reached midlog phase. Culture was pelleted at 3000 rpm for 5 min and cells were harvested by centrifugation. Pellet was washed with sterile water, and transferred to either synthetic complete medium or phosphate-deprived synthetic medium for different times. Lipids were extracted under acidic conditions using chloroform/methanol in a ratio of 1:2. The chloroform-soluble fraction was dried, suspended in HPLC grade methanol, and subjected to ESI-MS (Bruker Esquire 3000 Plus electrospray ion trap instrument). The sample (10 µl) was directly delivered into the ESI source through the polytetrafluoroethylene line at a rate of 4 µl/min. The ESI-MS settings used were as follows. The turboelectrospray ionization source was maintained at 260 °C, and data were collected in the negative ion mode from 420 to 450 m/z. Due to the lack of a proper internal standard with us, we added [3H]LPA to an equal amount of yeast cells; lipids were extracted in a similar way as that from samples subjected to ESI-MS; the efficiency of extraction was calculated to be 83–87%; and the procedure was monitored in every experiment. The efficiency of ionization was also almost the same every time. The experiments were repeated four times, and similar spectra were obtained in all of the cases.

In Vivo Labeling of E. coli Expressing the PHM8 Gene—E. coli cells were grown for more than three generations in the presence of [32P]orthophosphate until the $A_{600}$ reached 0.6. Culture was centrifuged at 5000 rpm for 10 min, resuspended in fresh Luria-Bertani medium, and induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 2 h. Lipids were extracted using chloroform/methanol in a ratio of 1:2 (v/v), separated by two-dimensional silica TLC, and analyzed by a PhosphorImager. E. coli expressing repressible acid phosphatase, Pho12p, was used as a control.

Cloning, Expression, and Purification of Recombinant Phm8p—Yeast genomic DNA was prepared as mentioned previously (19). The gene corresponding to PHM8 (YER037W) was amplified from the genomic DNA of S. cerevisiae using 5′-A-TATCTGAGATGACTATCGCTAACATGACTGAACATT-3′ and 5′-ATATGGTA CCTCATGATGACTGAACTATGTG-3′ as sense and antisense primers, respectively. The PCR mixture consisted of 100 ng of template, 10 pmol of sense and antisense primers, 0.2 mM dNTPs, 1.5 mM MgCl$_2$, 1 unit of XT-5 DNA polymerase (Bangalore Genei, India), and 1X reaction buffer. Amplification was carried out under the following conditions: initial denaturation of the template at 94 °C for 4 min followed by 30 cycles at 94 °C for 1 min (denaturation), 55 °C for 1 min (annealing), and 72 °C for 1 min (extension). The final extension was done at 72 °C for 20 min. The amplified product was cloned into pRSET C vector (Invitrogen) at KpnI and PstI restriction sites. The resultant plasmids containing the gene were confirmed by sequencing. The recombinant protein was expressed in E. coli Origami (DE3) pLysS cells (Novagen) by inducing with 0.5 mM IPTG followed by incubation for 2 h at 37 °C and purified by Ni$^{2+}$-NTA-agarose chromatography according to the manufacturer’s instructions. For expression of mutant enzymes, induction was carried out at 15 °C for 10 h after the addition of 0.5 mM IPTG. Before induction, the culture was maintained on ice for 30 min. Protein amounts were determined by using the Bio-Rad DC protein assay kit. Purified fractions containing eluted protein were analyzed by 10% SDS-PAGE followed by Coomassie Blue staining. Mutant proteins were purified in a similar manner as described for wild type.
Soluble LPA Phosphatase from Yeast

LPA Phosphatase Assay—LPA phosphatase assays were performed as described earlier (12). [1-oleoyl-9,10-3H]LPA (47 Ci/mmol) was suspended in 10 mM Triton X-100 and sonicated for 5 min in a sonic bath. The substrate stock solution was diluted 100-fold in the assay. The LPA phosphatase assay mixture consisted of 50 mM Mes (pH 6.5), 50 µM [3H]LPA (220,000 dpm/reaction), 2 mM MgCl2, 0.1 mM Triton X-100, and 1 µg of recombinant enzyme or 50 µg of yeast cytosol in a total volume of 100 µL. The incubation was carried out at 30 °C for the indicated time and the addition of 500 µL of 2% phosphoric acid followed by 600 µl of chloroform/methanol (1:2, v/v). Following lipid extraction, the lower chloroform-soluble fraction was separated by TLC on 250-µm silica gel G plates using chloroform/methanol/water (98:2:0.5, v/v/v) as the solvent system. The lipids were visualized by staining with iodine vapor, and spots of monoacylglycerol were scraped off for determination of radioactivity by liquid scintillation counting.

Other Enzyme Assays—50 µM diacylglycerol pyrophosphatase or sphingosine 1-phosphatase was incubated with the purified recombinant Phm8p, and the release of phosphate was monitored in the aqueous phase by using the malachite green method (20). Assay components and conditions are the same as described for the LPA phosphatase assay.

[glycerol-U-14C]Dipalmitoyl PA (50 µM, 165,000 dpm/assay) was incubated with the purified recombinant Phm8p for 30 min, and the reaction was stopped by the addition of 2% phosphoric acid followed by lipid extraction using chloroform/methanol in a ratio of 1:2 (v/v). The lower chloroform phase was spotted on TLC, and we estimated the formation of diacylglycerol in chloroform/methanol/water (98:2:0.5, v/v/v) as the solvent system.

The glycerol-3-phosphate phosphatase assay was carried out with the purified recombinant Phm8p by incubating with 50 µM [14C]glycerol-3-phosphate (110,000 dpm/reaction) for 30 min, and the reaction was stopped by the addition of 2% phosphoric acid. To this, an equal volume of chloroform was added and an aliquot of the supernatant was spotted on a TLC. The TLC was developed in 0.1M ammonium acetate in 67% ethanol using liquid scintillation counter.

Site-directed Mutagenesis—Site-directed mutagenesis was performed as reported previously (22). The following oligonucleotides were used as forward primers to amplify D58N, D60N, D58E, and D60E: 5'-CTTTAATATCAGTAAACACTTTGTACAGAAAGG-3', 5'-CTTTGATATC

RESULTS

Expression Analysis of Genes That Are Up-regulated as a Result of the PHO Regulatory Pathway—The expression connection available on the Saccharomyces Genome database (available on the World Wide Web) was used to retrieve microarray expression data. A total of 550 PHO-regulated genes were obtained when the search was limited to genes that are up-regulated 2-fold or more (data not shown). Of these 550 proteins, we identified 27 genes as phosphatases based on their reported functions or the corresponding domains present (supplemental Table 1). Of 27 phosphatases, the functions of three phosphatases have not been characterized so far. First is YBL112C that has a p-loop-containing nucleotide triphosphate hydrolase domain; second is YNL217W that has a metallodependent phosphoesterase domain that is commonly found in protein phosphatases or polyphosphatases of yeast; and third is YER037W (PHM8), which contains a HAD-like hydrolase domain. It has a JXDXT motif within the HAD-like domain, and the motif is characteristic of Mg2+-dependent phosphatases. Recently discovered lipid phosphate phosphatases (24, 25) also contain a HAD-like hydrolase domain. Our interest was to study the role of lipid phosphate phosphatases under
phosphate-deprived conditions. Therefore, we have initiated the study to decipher the role of Phm8p in *S. cerevisiae* under phosphate-deprived conditions.

**PHM8** gene is located on chromosome V of the yeast genome, and it does not contain any introns. This gene encodes for a protein product consisting of 321 amino acids. A Kyte-Doolittle plot with a window size of 19 amino acids (available on the World Wide Web) suggested that it has no possible transmembrane region and that it has no signal peptide sequence (data not shown).

**Phosphate Deprivation Affects Phospholipid Turnover**—To study whether the phosphatases that are up-regulated during phosphate starvation could play a role in phospholipid metabolism, we performed labeling studies to determine the turnover of phospholipids. Yeast cells were labeled with [³²P]orthophosphate/ml medium, such that final OD is 0.1, and then grown until the OD reached 1.0–1.2. Labeled cells were washed and resuspended either in SM + P, (A) or SM − P, (B) and chased for 6 h in the absence of radiolabel. At the end of the incubation period, lipids were extracted using chloroform/methanol (12, v/v) and resolved on two-dimensional silica TLC by chloroform/methanol/ammonia (65:25:5, v/v/v) as first dimension and chloroform/methanol/acetone/acetic acid/water (50:10:20:15:5, v/v/v/v/v) as second dimension solvent systems. Lipids are identified by numbers ranging from 1 to 12, phosphatidylinositol; 4, LPA; 7, phosphatidylinositol; 8, PC; 9, phosphatidylethanolamine; 10, cardiolipin; 11, PA, 1, 3, 5, and 6, unknown. O, origin. C, loss of phosphate in phospholipids was quantified by scraping the known phospholipid spots from the TLC plate and counted by a liquid scintillation counter using toluene-based scintillation fluid. inset, LPA turnover. Each data point represents the mean of three independent experiments ± S.D. D, the spot corresponding to LPA was scraped from a preparative TLC and analyzed by ESI-MS using a negative ion scan mode from 430 to 450 m/z.

**Identification of LPA Phosphatase**—[³²P]LPA hydrolysis was carried out *in vitro* using the cytosol of yeast grown in SM + P, and SM − P, as the enzyme source, and we found that there was a 2.07-fold increase in LPA-hydrolyzing activity in cytosol from yeast grown in phosphate-deprived synthetic medium. When magnesium was omitted from the assay mixture, there was a 50% reduction in the specific activity (Table 1). We detected a drastic increase in specific activity when cytosol was used and magnesium was included in the assay. From Table 1, it is obvious that magnesium-independent lipid phosphatases contribute to 58% of the total LPA phosphatase activity of the cell. LPP1 and DPP1 are membrane-bound lipid phosphatases that are known to dephosphorylate LPA in a magnesium-independent manner (14, 15). Phm8p contributes to 22% of the total LPA phosphatase activity in the cytosol under normal growth conditions, whereas, under phosphate-starved conditions, it contributes to more than 55% of the total LPA phosphatase activity in the cytosol. Phm8p is responsible for 75% of the magnesium-dependent LPA phosphatase activity in the cytosol.

In addition, we assayed for LPA-hydrolyzing ability of both wild type and *phm8Δ* cells, grown in normal SM + P, and phosphate-deprived synthetic medium (Table 1). The LPA phosphatase activity in the presence of magnesium was drastically reduced in *phm8Δ* mutant when compared with the wild type.
BY4741 cells, suggesting that PHM8 is responsible for the increased magnesium-dependent LPA phosphatase activity under phosphate-deprived conditions.

To authenticate the involvement of PHM8 in dephosphorylation of LPA during phosphate starvation, we have extracted lipids from the phm8/H9004 mutant yeast cells grown in complete synthetic medium and phosphate-deprived medium that were subjected to ESI-MS analyses (Fig. 3, A and B). It was observed under phosphate-deprived conditions that there was a 1.4-fold reduction in LPA levels.

To discover the function of PHM8 up-regulation during phosphate starvation, we have overexpressed it in E. coli and performed in vivo labeling with $^{32}$P orthophosphate followed by IPTG induction. We found a decrease in LPA concentration upon induction of Phm8p; E. coli cells harboring PHO12, a repressible acid phosphatase, were used as a control. There was no significant change in the amount of other phospholipids upon induction with IPTG (Fig. 4).

**TABLE 1**

| Strain type | Phosphate in synthetic medium | Specific activity | With Mg$^{2+}$ | Without Mg$^{2+}$ |
|-------------|-------------------------------|-------------------|-----------------|-------------------|
|             |                               | nmol/min/mg       |                 |                   |
| Wild type   |                               |                   |                 |                   |
| Cytosol     | +                             | 0.27 ± 0.018      | 0.15 ± 0.01     |
| Membranes   | +                             | 0.16 ± 0.01       | 0.10 ± 0.01     |
| Cytosol     | -                             | 0.56 ± 0.046      | 0.24 ± 0.01     |
| Membranes   | -                             | 0.13 ± 0.01       | 0.10 ± 0.008    |
| phm8Δ       |                               |                   |                 |                   |
| Cytosol     | +                             | 0.17 ± 0.009      | 0.14 ± 0.01     |
| Membranes   | +                             | 0.14 ± 0.01       | 0.10 ± 0.006    |
| Cytosol     | -                             | 0.25 ± 0.018      | 0.23 ± 0.012    |
| Membranes   | -                             | 0.11 ± 0.01       | 0.1 ± 0.008     |

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**FIGURE 2.** ESI-MS scan of chloroform-soluble fraction from yeast. Yeast cells were grown in synthetic complete medium until the OD reached 2.8–3.0. Cells (50 $A_{600}$) were pelleted, washed with sterile water, and suspended either in fresh SM + P, or SM − P, medium and grown at 30 °C for different times. Shown is an ESI-MS scan of lipids isolated from wild type yeast cells grown in SM + P (A) or SM − P (B) for 2 h.

**FIGURE 3.** ESI-MS scan of chloroform-soluble fraction from phm8Δ yeast. phm8Δ yeast was grown in the synthetic complete medium until the OD reached 2.8–3.0. The cells (60 $A_{600}$) were pelleted, washed with sterile water, and suspended either in fresh SM + P, or SM − P, medium and grown at 30 °C for 6 h. Shown is an ESI-MS scan of lipids (430–450 $m/z$) isolated from cells grown in SM + P (A) or SM − P (B).

**FIGURE 4.** ESI-MS scan of chloroform-soluble fraction from yeast. Yeast cells were grown in synthetic complete medium until the OD reached 2.8–3.0. Cells (50 $A_{600}$) were pelleted, washed with sterile water, and suspended either in fresh SM + P, or SM − P, medium and grown at 30 °C for different times. Shown is an ESI-MS scan of lipids isolated from wild type yeast cells grown in SM + P (C) or SM − P (D) for 6 h.
Soluble LPA Phosphatase from Yeast

Characterization of Recombinant Phm8p—Phm8p was overexpressed in E. coli by inducing with IPTG (Fig. 5A). Recombinant Phm8p was purified using Ni²⁺-NTA affinity chromatography (Fig. 5B), and it was confirmed by probing with anti-His tag monoclonal antibody (Fig. 5C). The purified protein showed Mg²⁺-dependent LPA phosphatase activity. We observed the time-dependent formation of [³²P]monoaoylglycerol when the recombinant enzyme was incubated in the presence of [³²P]LPA (Fig. 6A). The activity was greatly enhanced by the addition of magnesium, whereas cobalt and manganese showed 76 and 48% activity as compared with magnesium-dependent activity, respectively (Table 2). It exhibited a dose-dependent requirement for MgCl₂ with maximum activity at a final concentration of 2 mM (Fig. 6B). We did not observe LPA phosphatase activity in the absence of Mg²⁺ ions or in the presence of EDTA, Ca²⁺, or Zn²⁺. The enzyme reaction followed Michaelis-Menten kinetics with a $K_m$ value of 34 ± 2.07 μM (data not shown). The addition of Triton X-100 to the assay mixture resulted in an apparent inhibition of enzyme activity, characteristic of surface dilution kinetics (26). The activity was insensitive to phosphatase inhibitors like sodium fluoride and sodium orthovanadate at a concentration of 1 mM (data not shown).

Effect of pH on phosphatase activity was evaluated using different buffers of pH ranging from 4.0 to 8.8. The buffers used were acetate buffer (pH 4.0, 4.5, and 5.0), Mes buffer (pH 5.6 and 6.5), Tris buffer (pH 7.0, 7.5, 8.0, and 8.8). The enzyme was maximally active at pH 6.5 (Fig. 6C).

The addition of PA to the LPA phosphatase reaction was monitored to determine whether PA competitively inhibits the LPA hydrolysis reaction. Increasing concentrations of PA were added to the reaction mixture, and we found that there was no significant reduction in the enzyme activity (data not shown). To assess the substrate specificity of the Phm8p directly, we performed assays using radiolabeled lipid phosphates. These results indicated that the purified recombinant enzyme did not hydrolyze sphingosine 1-phosphate, diacylglycerol pyrophosphate, glycerol 3-phosphate, and PA (Fig. 6D), suggesting the specificity of this enzyme.

Overexpression of Phm8 in S. cerevisiae—The PHM8 gene was cloned into multi-copy plasmid pYES2 in KpnI and XhoI restriction sites. Transformants were confirmed by performing a colony PCR using vector-specific forward primer and PHM8-specific reverse primer (Fig. 7A). Induction was carried out by pelleting the cells, suspending them in 2% galactose-containing uracil-deprived medium, and allowing them to grow for 20 h. Overexpression of Phm8p was confirmed by Northern blotting (Fig. 7B). Cytosol from yeast overexpressing PHM8 showed a 5-fold increase in LPA phosphatase activity when compared with vector control (Fig. 7C). Upon overexpression of PHM8, we observed reduced amounts of LPA (Fig. 7D).

Identification of Catalytically Important Residues by Site-directed Mutagenesis—It is known that aspartate residues of the DXDXT motif (Fig. 8A) are important for phosphotransferases catalysis (27). The recently identified Saccharomyces phosphatidic acid phosphatase is also dependent on aspartate residues of the DXDXT motif for its activity (28). Aspartate residues at positions 58 and 60 were mutated to glutamate and asparagine, and these mutants (D58E, D58N, D60E, and D60N) were overexpressed in E. coli and purified using Ni²⁺-NTA-agarose chromatography (data not shown). The recombinant wild type and mutant purified proteins were assayed for their ability to hydrolyze LPA, D58E, D58N, and D60N were inactive, whereas D60E showed 33.5% of the activity of wild type Phm8p (Fig. 8B). These results indicated that Asp⁶⁰ plays a role in magnesium coordination, and catalysis proceeds through phosphoaspartate 58 intermediate as suggested previously (27).

**DISCUSSION**

Phosphatases are enzymes that catalyze the hydrolysis of phosphate esters from a variety of phosphorylated substrates, ranging from specific Thr/Ser residues of proteins to non-protein substrates, such as phospholipids. Phosphate ester hydrolysis is a hallmark of biochemical processes that are crucial in
signal transduction pathways and cell cycle regulation (29). During phosphate starvation, phosphatases play a major role in phosphate acquisition by hydrolyzing phosphorylated macromolecules (30). There are ~27 phosphatases that are up-regulated during phosphate starvation in yeast (supplemental Table 1). We have characterized Phm8p, and it contains a phosphatase motif within the HAD-like hydrolase domain, which is shown to be present in some recently identified lipid phosphatases (24, 25).

Lipids are important constituents of the cells, since they are integral components of the membranes. In general, many organisms alter their membrane lipid composition in response to chemical or physical changes in the environment (31). In plants, under phosphate starvation, neutral lipids are accumulated, and there is a change in the phospholipid metabolism (2, 4). In yeast, storage lipid accumulation was observed during inositol deficiency (32, 33), low temperature (34), and sporulation (35). The present study was undertaken to study the effect of phosphate deprivation on the regulation of phospholipid metabolism in *S. cerevisiae*, strain BY4741. We found that the level of LPA is decreased under phosphate deprivation, and this observation is in accordance with the report by Ramsay and Douglas (36). The decrease in the levels of LPA is due to the increase in LPA phosphatase activity in the cytosol of *S. cerevisiae*. Magnesium-independent soluble LPA phosphatase was reported from peanut (12) and bovine brain cytosol (13). The LPA phosphatase gene from bovine brain is similar to prostatic acid phosphatase that is involved in the regulation of mitochondrial phospholipid metabolism (37). The difference between the previously identified soluble LPA phosphatases and the yeast Phm8p is the requirement of magnesium ions for the enzyme activity.

Many genes are up-regulated during phosphate deprivation. The signal transduction pathway involved in the regulation of phosphate-responsive genes is complex, and it involves more
than 20 genes (38). Apart from PHO genes, PHM genes are also up-regulated during phosphate deprivation (7). We found that Phm8p is effective in dephosphorylating the LPA in a magnesium-dependent manner. This protein has the DXDXT motif that is found in the superfamily of Mg$^{2+}$-dependent phosphatases with diverse substrate specificities. This family comprises at least 10 different enzymes that are typically soluble proteins, acting either as monophosphate phosphatases or as phosphomutases (39).

Phm8p showed no activity in the absence of magnesium ions, which is similar to the previously identified DXDXT motif-containing enzymes (40, 41). The soluble epoxide hydrolase was shown to have lipid phosphate phosphatase activity that has been attributed to the presence of the N-terminal DXDXT motif, and the enzyme activity was not inhibited by sodium fluoride or sodium orthovanadate (24). A similar observation was made with LPA-hydrolyzing activity of Phm8p.

Soluble LPA Phosphatase from Yeast

**FIGURE 7.** Overexpression of PHM8 in S. cerevisiae. A, yeast transformation was confirmed by performing a PCR using PHM8-specific reverse primer and T7 promoter sequence as forward primer (in vector). Lane 1, pYES2; lane 2, pYES2-PHM8; lane 3, 1-kb DNA ladder. B, total RNA was isolated from plasmid-transformed yeast cells grown in galactose for 20 h and subjected to Northern blot hybridization with radiolabeled PHM8. Equal amount of RNA (20 µg) was loaded in all lanes. Lane 1, pYES2-transformed WT yeast cells induced with galactose; lane 2, pYES2-PHM8 overexpressed in phm8Δ yeast; lane 3, pYES2-PHM8 overexpressed in wild type cells. Lower panel, rRNA loading control. Positions of 18 and 28 S rRNAs are indicated. C, LPA phosphatase assay was done using cytosol of pYES2/pYES2-PHM8 overexpressing yeast. Shown are pYES2-induced (1) and pYES2-PHM8-induced cytosol, in the presence (2) and in the absence (3) of Mg$^{2+}$ in the assay. 4, assay with cytosol of pYES2-PHM8-induced Saccharomyces phm8Δ in the presence of Mg$^{2+}$. D, lipids were extracted from the yeast cells overexpressing Phm8p, and ESI-MS analysis was done. Yeast cells transformed with pYES2 served as a control. Values are the average of three independent experiments.

**FIGURE 8.** Role of aspartate residues of the DXDXT motif in LPA phosphatase activity. A, ClustalW alignment of the phosphatases belonging to the HAD family. Conserved amino acids are in boxes. Residues in **boldface italic** type are semiconserved. PSPase, phosphoserine phosphatase; HoPase, histidinol phosphatase; TPPase, trehalose-1-phosphate phosphatase; PGPase, phosphoglycerate phosphatase; EH, epoxide hydrolase; pah1p, phosphatidylycholine phosphatase. 8, mutational analysis of the DXDXT domain of Phm8p. WT, wild type enzyme. Values are the mean ± S.D. of three separate experiments, each performed in triplicate.
acting as a nucleophile (27). Mutating Asp^{59} to either glutamate or asparagine in Phm8p resulted in complete loss of LPA-hydrolyzing activity, suggesting that the DXDXT motif is responsible for the phosphatase activity and that this activity is not due to the minor contaminant proteins found after Ni^{2+}-NTA purification. However, the D60E mutant retained a partial activity of the enzyme. An in vitro assay for LPA hydrolysis of the \textit{phm8Δ} mutant showed a reduced level of LPA-hydrolyzing activity as compared with wild type. This finding suggested that Phm8p is indeed responsible for the increased LPA phosphatase activity during phosphate starvation. The \textit{phm8Δ} mutant did not show a complete reduction in LPA phosphatase activity, suggesting that apart from Phm8p, there is another LPA-degrading phosphatase(s) that could be up-regulated during phosphate deprivation. Our data suggest that Phm8p possibly plays a role in the signal transduction pathway involved in regulation of phosphate metabolism. It would be of interest to see whether Phm8p has any role in the regulation of lipid metabolism.

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