Mg\(^{2+}\)-dependent phosphatidate (PA) phosphatase (3-sn-phosphatidate phosphohydrolase, EC 3.1.3.4) catalyzes the dephosphorylation of PA to yield diacylglycerol and Pi. In this work, we identified the *Saccharomyces cerevisiae* PAH1 (previously known as SMP2) gene that encodes Mg\(^{2+}\)-dependent PA phosphatase using amino acid sequence information derived from a purified preparation of the enzyme (Lin, Y.-P., and Carman, G. M. (1989) J. Biol. Chem. 264, 8641–8645). Overexpression of PAH1 in *S. cerevisiae* directed elevated levels of Mg\(^{2+}\)-dependent PA phosphatase activity, whereas the *pah1Δ* mutation caused reduced levels of enzyme activity. Heterologous expression of PAH1 in *Escherichia coli* confirmed that Pa1p is a Mg\(^{2+}\)-dependent PA phosphatase enzyme and showed that its enzymological properties were very similar to those of the enzyme purified from *S. cerevisiae*. The PAH1-encoded enzyme activity was associated with both the membrane and cytosolic fractions of the cell, and the membrane-bound form of the enzyme was salt-extractable. Lipid analysis showed that mutants lacking PAH1 accumulated PA and had reduced amounts of diacylglycerol and its derivative triacylglycerol. The PAH1-encoded Mg\(^{2+}\)-dependent PA phosphatase shows homology to mammalian lipin, a fat-regulating protein whose molecular function is unknown. Heterologous expression of human LPIN1 in *E. coli* showed that lipin 1 is also a Mg\(^{2+}\)-dependent PA phosphatase enzyme.

PA\(^{2+}\) phosphatase (3-sn-phosphatidate phosphohydrolase, EC 3.1.3.4) catalyzes the dephosphorylation of PA yielding DAG and Pi (1). In the yeast *Saccharomyces cerevisiae*, the DAG generated in this reaction is utilized for the synthesis of phosphatidylcholine and phosphatidylethanolamine via the CDP-choline and CDP-ethanolamine branches of the Kennedy pathway and for the synthesis of TAG (2–6). In higher eukaryotes, PA phosphatase also plays a role in lipid signaling as part of the phospholipase D-PA phosphatase pathway for the generation of DAG from phosphatidylcholine (7–9). The importance of PA phosphatase in lipid signaling is further emphasized by its role in attenuating the bioactive functions of PA (9–11). Mg\(^{2+}\)-dependent and -independent forms of PA phosphatase have been identified in *S. cerevisiae* (12, 13). Nearly all Mg\(^{2+}\)-independent PA phosphatase activity is encoded by the DPP1 (14) and LPP1 (15) genes, with DPP1 being the major contributor of this activity (15). The DPP1- and LPP1-encoded enzymes are integral membrane proteins localized to the vacuole (16, 17) and Golgi (18) compartments of the cell, respectively. These enzymes have broad substrate specificity; in addition to PA, they utilize a variety of lipid phosphate substrates including diacylglycerol pyrophosphate, lyso-PA, and isoprenoid phosphates (14, 15, 19–21). The DPP1 and LPP1 genes are not essential under standard laboratory conditions (14, 15). Mutants defective in the DPP1 and LPP1 genes do not exhibit any growth defect or morphological abnormalities that shed light on the physiological roles of their gene products (14, 15). The DPP1-encoded phosphatase enzyme regulates the cellular levels of PA and diacylglycerol pyrophosphate in vacuole membranes of zinc-depleted cells, but the physiological significance of this regulation is unclear (17). It is unknown whether the LPP1 gene product controls phospholipid composition in Golgi membranes. Although the DPP1- and LPP1-encoded Mg\(^{2+}\)-independent PA phosphatase activities may regulate specific pools of PA in the vacuole and Golgi, respectively, they are not responsible for the *de novo* synthesis of phospholipids and TAG that presumably occurs in the ER.

The Mg\(^{2+}\)-dependent PA phosphatase is postulated to be responsible for the DAG needed for the synthesis of phospholipids and TAG in *S. cerevisiae* (6, 12). Cytosolic and membrane-associated forms of the Mg\(^{2+}\)-dependent PA phosphatase have been purified and characterized with respect to their enzymological and regulatory properties (12, 22–29). Unlike the Mg\(^{2+}\)-independent forms of PA phosphatase, the Mg\(^{2+}\)-dependent forms of the enzyme are specific for PA and require Mg\(^{2+}\) ions for catalytic activity (22, 24, 29). However, genes encoding Mg\(^{2+}\)-dependent PA phosphatase enzymes have not been identified from *S. cerevisiae* or from any organism (12). Because mutants defective in these enzymes are not available, it has not been established whether the Mg\(^{2+}\)-dependent PA phosphatases previously isolated from *S. cerevisiae* (22, 24, 29) are in fact responsible for *de novo* lipid synthesis.

In this paper we report for the first time the identification of the *S. cerevisiae* PAH1 (previously known as SMP2) gene encoding a Mg\(^{2+}\)-dependent PA phosphatase enzyme. Lipid analysis of a *pah1Δ* mutant showed that the Mg\(^{2+}\)-dependent PA phosphatase is indeed a relevant enzyme responsible for *de novo* lipid synthesis in *S. cerevisiae*. Moreover, we showed that lipin 1, a mammalian fat-regulating protein that is homologous to Pah1p (30), exhibits Mg\(^{2+}\)-dependent PA phosphatase activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were reagent grade. Growth medium supplies were purchased from Difco. Restriction endonucleases, modifying enzymes, and recombinant Vent DNA polymerase were purchased from New England Biolabs. DNA purification kits and Ni\(^{2+}\)-NTA-agarose resin were purchased from Qiagen. Oligonucleotides were prepared by Genosys Biotechnologies, Inc. The Yeast Maker yeast transformation kit was purchased from Clontech. Radiochemicals were purchased from PerkinElmer Life Sciences. Nucleotides, phenylmeth-
**TABLE 1**

| Strain or plasmid | Genotype or relevant characteristics | Source or Ref. |
|-------------------|--------------------------------------|---------------|
| E. coli DH5α | Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK- mB- sB-) phoA supE44 Δ(λ-934)F' lac-proT1 ΔZam15 (pR plasmids) | Novagen |
| BL21(DE3)pLysS | ompT hsdR2 (rK- mB-) gal dcm (DE3) pLysS | Novagen |

**S. cerevisiae**

| Strain | Genotype or relevant characteristics | Source or Ref. |
|--------|-------------------------------------|---------------|
| BY4742 | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | Invitrogen |
| W303-1A | ade2-1 can1-100 his3Δ-11,15 leu2-3,112 trp1Δ-1 ura3Δ-1 | This study |
| GHY57 | ade2-1 can1-100 his3Δ-11,15 leu2-3,112 trp1Δ-1 ura3Δ-1 pahΔ::URA3 | This study |
| TBY1 | ade2-1 can1-100 his3Δ-11,15 leu2-3,112 trp1Δ-1 ura3Δ-1 pahΔ::URA3 | This study |
| GHY58 | ade2-1 can1-100 his3Δ-11,15 leu2-3,112 trp1Δ-1 ura3Δ-1 pahΔ::URA3 | This study |
| MC13 | ade2-1 can1-100 his3Δ-11,15 leu2-3,112 ura3Δ-1 pahΔ::URA3 | This study |
| SH1100 | ade2-1 can1-100 his3Δ-11,15 leu2-3,112 ura3Δ-1 opi1Δ::KanMX | This study |

**Plasmids**

| Plasmid | Description | Source |
|---------|-------------|--------|
| YEp351 | Multicopy E. coli yeast shuttle vector with LEU2 | 79 |
| pRS406 | Integrating E. coli yeast shuttle vector with URA3 | 80 |
| pGH311 | PAH1 gene inserted into YEp351 | This study |
| pGH312 | PAH1<sup>′</sup> gene inserted into YEp351 | This study |
| pGH317 | pahΔ::URA3 inserted into pGH311 | This study |
| pET-15b | E. coli expression vector with N-terminal His<sub>6</sub> tag fusion | Novagen |
| pGH313 | PAH1 coding sequence inserted into pET-15b | This study |
| pGH318 | LPIN1 coding sequence inserted into pET-15b | This study |

**PAH1-encoded Mg<sup>2+</sup>-dependent PA Phosphatase**

LPIN1, E. coli BL21(DE3)pLysS cells bearing pGH313 and pGH318, respectively, were grown to A<sub>600</sub> = 0.5 at room temperature in 500 ml of LB medium containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml). The culture was incubated for 1 h with 1 μl isopropyl β-D-thiogalactopyranoside to induce the expression of His<sub>6</sub>-tagged Pah1p and lipin 1.

**DNA Manipulations, Amplification of DNA by PCR, and DNA Sequencing**—Standard methods were used to isolate plasmid DNA and yeast genomic DNA and for manipulation of DNA using restriction enzymes, DNA ligase, and modifying enzymes (32). Yeast (35) and E. coli (32) transformations were performed by standard protocols. PCR reactions were optimized as described by Innis and Gelfand (36). DNA sequencing reactions were performed by the dyeodeoxy method using Taq DNA polymerase (32) and analyzed by automated DNA sequencer.

**Construction of Plasmids**—The plasmids used in this work are listed in Table 1. The S. cerevisiae PAH1 gene (SMP2/YMR165C) was cloned by PCR. A 3.8 kb DNA fragment that contains the entire coding sequence (2.6 kb) of PAH1, the 5′-untranslated region (0.7 kb), and the 3′-untranslated region (0.5 kb) was amplified from the genomic DNA of S. cerevisiae strain BY4742. The PAH1 DNA fragments were digested with XbaI/SphI and inserted into plasmid YEp351 at the same restriction enzyme sites. The multicopy plasmid containing PAH1 was named pGH311. The PAH1 gene was used to construct PAH1<sup>HA</sup>, in which the sequence for an HA epitope tag (YPYDVPDYA) was located after the start codon. The 0.7- and 3.1-kb PAH1 DNA fragments that contain the HA tag at the 3′ and 5′ ends, respectively, were amplified by PCR. These DNA fragments were digested with XbaI/AatII and AatII/SphI, respectively, and inserted into YEp351 at the XbaI/SphI sites. The multicopy plasmid containing PAH1<sup>HA</sup> was named pGH312. For expression of PAH1 in E. coli, the entire coding sequence of PAH1 was amplified by PCR using plasmid pGH311 as a template. The PCR product (~2.6 kb) was digested with HindIII to produce 0.6- and 2.0-kb DNA fragments, which were then digested with NdeI and XhoI, respectively. The NdeI-HindIII and HindIII-XhoI DNA fragments were inserted into pET-15b at the NdeI/XhoI sites. The E. coli expression plasmid containing the His<sub>6</sub>-tagged PAH1 was named pGH313. For expression of human LPIN1 (accesion number NM_145693) in E. coli, the entire coding sequence of the gene was amplified from a full-length LPIN1 cDNA clone (Origene Technologies, Inc.) by PCR using primers with add-on restriction enzyme sites (MluI before start codon/XhoI after stop codon). The PCR product (2.7 kb) was digested with MluI, filled with Klenow, and digested with XhoI. The LPIN1 DNA fragment was ligated with pET-15b that was digested with NdeI, filled with Klenow, and digested with XhoI. The E. coli expression plasmid containing the His<sub>6</sub>-tagged LPIN1 was named pGH318.

**Construction of the pahΔ Mutant and the pahΔ dpp1Δ ipp1Δ Triple Mutant—URA3 DNA (1.4 kb) was amplified from plasmid pRS406 by PCR using primers with add-on restriction enzyme sites. The PCR products were digested with Thh111 and Spel and inserted into the plasmid pGH311 that was digested with the same restriction enzymes to remove 80% of the PAH1 coding sequence. The resulting plasmid, which contains a 3-kb PAH1 deletion cassette (pahΔ::URA3), was named pGH317. Deletion of the PAH1 gene in the yeast chromosome was performed by the method of one-step gene replacement (37). The PAH1 deletion cassette was released from plasmid pGH317 by digestion with XbaI and Spel and used to transform strains W303-1A and TBY1. The resulting transformants were selected on SC-uracl medium. Disruption of the PAH1 gene in uracil prototrophs was examined by PCR analysis of genomic DNA using primers that flank the inserted URA3 gene.
Preparation of the Cytosolic and Membrane Fractions from S. cerevisiae—All steps were performed at 4 °C. Yeast cells were suspended in 50 mM Tris-HCl, pH 7.5, 0.3 M sucrose, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin. Cells were disrupted with glass beads (0.5 mm diameter) using a Biospec Products Mini-BeadBeater-8 as described previously (38). Unbroken cells and cell debris were removed by centrifugation at 1,500 × g for 10 min. The cell lysate was centrifuged at 100,000 × g for 1 h to separate cytosolic (supernatant) from membrane fractions (pellet). The membranes were suspended in the same buffer used to disrupt cells. Protein concentration was estimated by the method of Bradford (39) using bovine serum albumin as the standard.

Purification of His6-tagged Pah1p and Human Lipin 1—All steps for protein purification were performed at 4 °C. E. coli cells expressing His6-tagged Pah1p and lipin 1 were washed once with 20 mM Tris-HCl, pH 8.0, buffer and suspended in 20 ml of 20 mM Tris-HCl, pH 8.0, buffer containing 0.5 mM NaCl, 5 mM imidazole, and 1 mM 2-mercaptoethanol. Cells were disrupted by a freeze-thawing cycle and by two passages through a French press at 20,000 pounds/square inch. Unbroken cells and cell debris were removed by centrifugation at 12,000 × g for 30 min at 4 °C. The supernatant (cell lysate) was gently mixed with 2 ml of 50% slurry of Ni2+-NTA-agarose for 2 h. The Ni2+-NTA-agarose/enzyme mixture was packed in a 10-ml Poly-Prep column and washed with 20 ml of 20 mM Tris-HCl, pH 8.0, buffer containing 0.5 mM NaCl, 45 mM imidazole, 10% glycerol, and 7 mM 2-mercaptoethanol. His6-tagged proteins were then eluted from the column in 1 ml fractions with a total volume of 0.1 ml. Diacylglycerol pyrophosphate phosphatase protein purification were performed at 4 °C.

Enzyme Assays—Mg2+-dependent PA phosphatase activity was measured by following the release of water-soluble 32Pi from chloroform-soluble [32P]PA (10,000 cpm/nmol) as described by Carman and Lin (42). The reaction mixture contained 50 mM Tris-HCl buffer, pH 7.5, 1 mM MgCl2, 0.2 mM PA, 2 mM Triton X-100, and enzyme protein in a total volume of 0.1 ml. Diacylglycerol pyrophosphate phosphatase activity was measured by following the release of water-soluble 32Pi from chloroform-soluble [β-32P]diacylglycerol pyrophosphate (10,000 cpm/nmol) as described by Wu et al. (19). The reaction mixture contained 50 mM citrate buffer, pH 5.0, 0.1 mM diacylglycerol pyrophosphate, 2 mM Triton X-100, and enzyme protein in a total volume of 0.1 ml. All enzyme assays were conducted in triplicate at 30 °C. The average standard deviation of the assays was ± 5%. The 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 per minute.

Labeling and Analysis of Lipids—Steady-state labeling of phospholipids and neutral lipids with 32P and [2-14C]acetate, respectively, were performed as described previously (43–46). Lipids were extracted from labeled cells by the method of Bligh and Dyer (47). Phospholipids were analyzed by two-dimensional thin-layer chromatography on silica gel plates using chloroform/methanol/ammonium hydroxide/water (45:25:2.3, v/v) as the solvent system for dimension one and chloroform/methanol/glacial acetic acid/water (32:4:5:1, v/v) as the solvent system for dimension two (48). Neutral lipids were analyzed by one-dimen-sional thin-layer chromatography on silica gel plates using the solvent system hexane/diethyl ether/glacial acetic acid (40:10:1, v/v) (49). The identity of the labeled lipids on thin-layer chromatography plates was confirmed by comparison with standards after exposure to iodine vapor. Radiolabeled lipids were visualized by phosphorimaging analysis. The relative quantities of labeled lipids were analyzed using ImageQuant software.

Data Analysis—Kinetic data were analyzed with the EZ-FIT enzyme kinetic model-fitting program (50), and statistical analyses were performed with SigmaPlot software. p values < 0.05 were taken as a significant difference.

RESULTS

Identification of the S. cerevisiae PAH1 Gene Encoding a Mg2+-dependent PA Phosphatase Enzyme—Mg2+-dependent PA phosphatase was partially purified (through the Mono Q chromatography step) from sodium cholate-treated membranes as described by Lin and Carman (22). An SDS-polyacrylamide gel slice containing a protein with a molecular mass of 91 kDa was subjected to trypsin digestion followed by amino acid sequence analysis of peptide fragments by matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry.3 Unambiguous amino acid sequence information obtained from 23 peptides (Fig. 1) matched perfectly with the deduced amino acid sequence of SMP2 (YMR165C) in the Saccharomyces Genome Data Base. We renamed this gene PAH1 (phosphatidic acid phosphohydrolase) because its protein product has the molecular function of a PA phosphatase encoded by the PAH1 gene.

3 The amino acid sequence analysis was performed at the Center for Advanced Proteomics Research at the University of Medicine and Dentistry of New Jersey in Newark.
FIGURE 2. The effects of the pah1Δ mutation and PAH1 gene overexpression on Mg²⁺-dependent PA phosphatase activity in S. cerevisiae and immunoblot analysis of Pah1pHA. A, cytosolic and membrane fractions were prepared from the indicated cells at the exponential phase of growth and assayed for Mg²⁺-dependent PA phosphatase activity. The data shown were determined from triplicate enzyme determinations ± S.D. B, a sample (15 μg) of cell extract derived from dpp1Δ lpp1Δ cells overexpressing the PAH1HA gene was subjected to immunoblot analysis using anti-HA antibodies (1 μg/ml). The positions of Pah1pHA and molecular mass standards are indicated in the figure.

PAH1-encoded Mg²⁺-dependent PA Phosphatase

The PAH1 sequence is found on chromosome XIII and does not have any sequence motifs suggesting introns in the gene, and its deduced protein product consists of 862 amino acids. Pah1p contains a conserved haloacid dehalogenase (HAD)-like domain (51, 52) in the middle of the protein sequence (Fig. 1), which contains a DXDXT (residues 398–402) motif that is found in a superfamily of Mg²⁺-dependent phosphatases enzymes with diverse substrate specificity (51, 52).

To examine the hypothesis that PAH1 encodes a Mg²⁺-dependent PA phosphatase, we examined the levels of the enzyme activity from cell extracts derived from exponential phase cells. In the pah1Δ mutant, the Mg²⁺-dependent PA phosphatase activity was reduced by 34% when compared with the activity found in the wild type parent (Fig. 2A). We also examined the effect of the pah1Δ mutation on Mg²⁺-dependent PA phosphatase activity in the dpp1Δ lpp1Δ mutant to eliminate the contributions of the DPP1-encoded and LPP1-encoded Mg²⁺-independent PA phosphatase activities that are still active under the assay conditions (e.g. 1 mM MgCl₂) used for the Mg²⁺-dependent activity (22, 24, 29). The Mg²⁺-dependent PA phosphatase activity in the cell extract of pah1Δ dpp1Δ lpp1Δ triple mutant cells was 30% lower that the activity in the dpp1Δ lpp1Δ double mutant (Fig. 2A). The remaining Mg²⁺-dependent PA phosphatase activity in the triple mutant must be attributed to yet another gene that codes for a PA phosphatase enzyme.

The multiplicity plasmid containing the PAH1 gene directed a 4-fold overexpression of PA phosphatase activity when compared with dpp1Δ lpp1Δ cells not bearing the plasmid (Fig. 2A). As would be expected, the PA phosphatase activity directed by the PAH1 gene was dependent on the presence of Mg²⁺ ions in the assay buffer. A PAH1Δ allele was constructed and cloned into a multiplicity plasmid for identification of Pah1p by immunoblotting. The HA-tagged version of the enzyme was functional and exhibited the same levels of Mg²⁺-dependent PA phosphatase activity in the dpp1Δ lpp1Δ mutant as the untagged enzyme. Immunoblot analysis showed that the HA-tagged PA phosphatase (Pah1pHA) migrated as a 124-kDa protein under SDS-PAGE (Fig. 2B).

The 91-kDa protein that was used to identify the PAH1 gene was isolated from the membrane fraction of yeast (22). Yet, Pah1p does not contain any transmembrane-spanning regions. Localization studies with a Pah1p fused with green fluorescent protein indicated that the Pah1p is present throughout the cytoplasm (18). Given this information, the association of Mg²⁺-dependent PA phosphatase activity with the cytosolic and membrane fractions of the cell was examined. As described previously (14, 15), most (66%) of the membrane-associated PA phosphatase activity in wild type cells was attributed to the DPP1 and LPP1 gene products (Fig. 2A). Analysis of Mg²⁺-dependent PA phosphatase activity in the pah1Δ and pah1Δ dpp1Δ lpp1Δ mutants and in the dpp1Δ lpp1Δ mutant overexpressing the PAH1HA gene showed that the PAH1-encoded enzyme was found in both the cytosolic and membrane fractions (Fig. 2A). About 70% of the PAH1-encoded Mg²⁺-dependent PA phosphatase present in the membrane fraction was extracted with 0.5 M NaCl (Fig. 3). This result indicated that the PAH1-encoded enzyme could associate with membranes as a peripheral membrane protein (53). The membrane-associated Mg²⁺-dependent PA phosphatase activity present in the pah1Δ dpp1Δ lpp1Δ triple mutant was also salt-extractable (Fig. 3).

Mg²⁺-dependent and -independent forms of PA phosphatase have been characterized as being sensitive or insensitive to the thiolreactive agent NEM (12, 20). We examined the effect of NEM on the Mg²⁺-dependent PA phosphatase activity in the cytosolic fraction of wild type, dpp1Δ lpp1Δ, and pah1Δ dpp1Δ lpp1Δ cells. NEM inhibited the PA phosphatase activity in wild type cells and in dpp1Δ lpp1Δ mutant cells by 26 and 27%, respectively (Fig. 4). Analysis of Mg²⁺-dependent PA phosphatase activity present in the pah1Δ dpp1Δ lpp1Δ triple mutant, 90% of the PA phosphatase was inhibited by NEM (Fig. 4). These results indicated that the PAH1-encoded PA phosphatase was an NEM-insensitive enzyme whereas the remaining PA phosphatase activity in the triplet mutant was an NEM-sensitive enzyme.

Heterologous Expression of the PAH1-encoded Mg²⁺-dependent PA Phosphatase in E. coli—The overexpression of Mg²⁺-dependent PA phosphatase activity in dpp1Δ lpp1Δ cells bearing the PAH1 gene on a multicopy plasmid indicated that PAH1 encodes a Mg²⁺-dependent PA phosphatase enzyme. However, this result did not rule out the possibility that the PAH1 gene was a regulatory gene whose product controlled the expression or activities of PA phosphatase enzymes. We used heterologous expression of the yeast PAH1 gene in E. coli to test the hypothesis that the PAH1 gene was the structural gene encoding a Mg²⁺-dependent PA phosphatase enzyme. The purified His₆-tagged Pah1p migrated as a 114-kDa protein upon SDS-PAGE (Fig. 5A). This protein catalyzed the dephosphorylation of PA in a protein concentration-dependent manner (Fig. 5B). The specific activity of the recombinant PA phosphatase enzyme was 3,000 nmol/min/mg. If we assume that the specific activity of the PAH1-encoded Mg²⁺-dependent PA phosphatase in the cell extract of yeast is 0.5 nmol/min/mg (based on the data in Figs. 2 and 4), then the specific activity of the purified recombinant enzyme represents a 6,000-fold enrichment of the enzyme.

We examined the basic enzymological properties of the purified recombinant Mg²⁺-dependent PA phosphatase enzyme. Optimum enzyme activity was found at pH 7.5 (Fig. 6A). No enzyme activity was
PAH1-encoded Mg\textsuperscript{2+}-dependent PA Phosphatase

![Graph showing the effect of NaCl on the membrane association of Mg\textsuperscript{2+}-dependent PA phosphatase activity.](image)

![Graph showing the effect of NEM on the Mg\textsuperscript{2+}-dependent PA phosphatase activity in S. cerevisiae wild type, dpp1\(\Delta\) lpp1\(\Delta\), and pah1\(\Delta\) dpp1\(\Delta\) lpp1\(\Delta\) cells.](image)

observed when MgCl\textsubscript{2} was omitted from the standard reaction mixture (Fig. 6B). PA phosphatase activity exhibited a dose-dependent requirement for MgCl\textsubscript{2} with maximum activity at a final concentration of 1 mM (Fig. 6B). NEM (1–20 mM) did not affect the activity of the enzyme. The effect of Triton X-100 on Mg\textsuperscript{2+}-dependent PA phosphatase activity is shown in Fig. 6C. The addition of Triton X-100 to the assay mixture resulted in the apparent inhibition of activity characteristic of surface dilution kinetics (54). The function of Triton X-100 in the assay for Mg\textsuperscript{2+}-dependent enzymes is to form a mixed micelle with the lipid substrate providing a surface for catalysis (54). Since the PAH1-encoded Mg\textsuperscript{2+}-dependent PA phosphatase exhibited surface dilution kinetics, the kinetic analysis of the enzyme was performed using Triton X-100/PA-dependent PA phosphatase activity (22, 23) as well as many other lipid-dependent enzymes to utilize a variety of lipid phosphate substrates such as diacylglycerol pyrophosphate (12, 14, 15, 20). As described previously for the Mg\textsuperscript{2+}-dependent PA phosphatase purified from yeast (19), the PAH1-encoded enzyme did not utilize diacylglycerol pyrophosphate as a substrate.

Phenotypic Properties of the pah1\(\Delta\) Mutant—A mutation in PAH1 (SMP2) was first identified in a screen for mutants that exhibit increased stability of heterologous plasmids (55). More recently, Santos-Rosa et al. (56) have shown that pah1\(\Delta\) (smp2\(\Delta\)) mutants have enlarged, irregularly shaped nuclei with projections that associate with the peripheral ER. This phenotype has been attributed to increased membrane phospholipid synthesis because the INO1, INO2, and OPE3 phospholipid synthesis genes are derepressed in the pah1\(\Delta\) (smp2\(\Delta\)) mutant background (56). The derepression of INO1 in the pah1\(\Delta\) (smp2\(\Delta\)) mutant prompted us to examine the mutant for the inositol excretion phenotype (34). Inositol excretion is due to the overexpression of INO1-encoded inositol-3-phosphate synthase activity and massive production of inositol (57–59). However, the pah1\(\Delta\) mutant did not exhibit the inositol excretion phenotype. As described previously (56), the pah1\(\Delta\) mutant grew more slowly than wild type cells at 30 °C and exhibited a temperature-sensitive phenotype at 37 °C (Fig. 7). Interestingly, the dpp1\(\Delta\) lpp1\(\Delta\) double mutant, which is defective in nearly all of the Mg\textsuperscript{2+}-independent PA phosphatase activity in yeast (15), grew equally...
PAH1-encoded Mg$^{2+}$-dependent PA Phosphatase

as well as wild type cells at both 30 and 37 °C (Fig. 7). The dpp1Δ lpp1Δ mutations, however, slightly exacerbated the temperature-sensitive phenotype of the pah1Δ mutation (Fig. 7).

We questioned whether the pah1Δ mutation would affect the cellular levels of PA. In these experiments, cells were grown to the exponential and stationary phases of growth in medium lacking inositol. Both growth phases were examined because Mg$^{2+}$-dependent PA phosphatase activity is elevated in stationary phase cells (46, 60). Inositol was omitted from the growth medium to preclude the regulatory effects that aninositol precursor has on phospholipid synthesis (5, 61). Phospholipids were extracted, separated by two-dimensional thin-layer chromatography, and the images were subjected to ImageQuant analysis. The percentages shown for the individual phospholipids were normalized to the total $^{32}$P-labeled chloroform-soluble fraction that included sphingolipids and unidentified phospholipids. Each data point represents the average of three experiments ± S.D. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidate.

DAG is used by DAG acyltransferase enzymes to produce TAG (6, 62, 63). If the PAH1-encoded Mg$^{2+}$-dependent PA phosphatase contributes to the production of this pool of DAG, then the pah1Δ mutation might be expected to affect TAG composition. This question was addressed by labeling cells with [2-14C]acetate followed by the extraction and analysis of neutral lipids by thin-layer chromatography. We analyzed TAG in both the exponential and stationary phases of growth because the level of TAG is elevated in stationary phase cells (60, 64). In exponential phase cells, the TAG content of the pah1Δ mutant was 62% lower than that of the wild type parent (Fig. 9A). The effect of the pah1Δ mutation on TAG content was even more dramatic in stationary phase cells; TAG levels dropped by 92% when compared with the control (Fig. 9B). That the defect in PA phosphatase activity in the pah1Δ mutant was responsible for the reduction in TAG content was supported by the increases in phosphatidylethanolamine (50%) and phosphatidylinositol (80%) (Fig. 8A). Similar effects on phospholipid composition were observed in the pah1Δ dpp1Δ lpp1Δ triple mutant (Fig. 8A).
have a major effect on the changes in TAG and DAG that were brought
about by the pah1Δ mutation itself. However, some of the effects of the
pah1Δ mutation on sterols and fatty acids were enhanced by the dpp1Δmutations (Fig. 9). The dpp1Δ lpp1Δ mutations by themselves
did not have a major effect on neutral lipid composition in either the
exponential or stationary phases of growth (Fig. 9).

**Heterologous Expression of the Human LPIN1 cDNA in E. coli**

**Identification of Lipin 1 as a Mg$^{2+}$-dependent PA Phosphatase Enzyme**—The protein product of the human LPIN1 gene (i.e. lipin 1) shares sequence homology with yeast Pah1p (30, 56). Although the
molecular function of lipin 1 is unknown, it is known that this protein in
mice plays a major role in fat homeostasis (30, 65–69). Accordingly, we
questioned the possibility that lipin 1 might be a Mg$^{2+}$-dependent PA phosphatase enzyme. To test this hypothesis, we expressed human LPIN1 cDNA in E. coli. Purified His$_{6}$-tagged human lipin 1 was assayed for Mg$^{2+}$-dependent PA phosphatase activity. The results of this assay showed that human lipin 1 was in fact a Mg$^{2+}$-dependent PA phosphatase enzyme. The specific PA phosphatase activity of the purified protein
was 1,600 nmol/min/mg. This level of activity was comparable with that of the purified recombinant PAH1-encoded Mg$^{2+}$-dependent PA phosphatase enzyme. The enzymological characterization of the LPIN1-encoded Mg$^{2+}$-dependent PA phosphatase will be the subject of separate report.

**DISCUSSION**

The Mg$^{2+}$-dependent form of PA phosphatase is postulated to be the
enzyme involved in the de novo synthesis of TAG and phospholipids (via
the Kennedy pathway) in *S. cerevisiae* (6, 12). However, this notion has
not been established because a gene encoding this enzyme is unknown.
In this work using a reverse genetic approach, we identified PAH1 as the
gene encoding a Mg$^{2+}$-dependent PA phosphatase enzyme. Overexpression
of the PAH1 gene in *S. cerevisiae* resulted in elevated levels of Mg$^{2+}$-dependent PA phosphatase activity, whereas the deletion of the
gene resulted in the reduction of this enzyme activity. Moreover, cells
containing the pah1Δ mutation accumulated PA and had reduced amounts of DAG and its acylated derivative TAG. The effects of the pah1Δ mutation on TAG content were most evident in the stationary
phase of growth where the synthesis of TAG predominates over the
synthesis of phospholipids (60, 64). Likewise, the pah1Δ mutation
showed the most striking effects on phospholipid composition in the
exponential phase of growth where the synthesis of phospholipids dom-
ninates over TAG synthesis (60, 64). The heterologous expression of the
*S. cerevisiae* PAH1 gene in *E. coli* confirmed that Pah1p possessed Mg$^{2+}$-dependent PA phosphatase activity. Moreover, the enzymological prop-
erties of the recombinant Mg$^{2+}$-dependent PA phosphatase were very
similar to those of the 91-kDa enzyme previously purified from *S. cer-
veisiae* (22, 23, 25). Collectively, these data provided conclusive evidence that the *S. cerevisiae* PAH1 gene is a bona fide structural gene encoding
a Mg$^{2+}$-dependent PA phosphatase in *S. cerevisiae* and that this enzyme
does in fact generate DAG for lipid synthesis.

The 91-kDa form of Mg$^{2+}$-dependent PA phosphatase used to iden-
tify the PAH1 gene was a proteolytic product of a larger sized enzyme
(24). In fact, the sequence information derived from the 91-kDa enzyme lacked sequences at the C-terminal end of the protein (Fig. 1). The
predicted size of Pah1p is 95 kDa. However, Pah1p expressed in *S. cer-
veisiae* migrated as a 124-kDa protein upon SDS-PAGE, whereas the
protein expressed in *E. coli* migrated as a 114-kDa protein. The reason
for the slow migration of *E. coli*-expressed Pah1p upon SDS-PAGE is
unclear, but the differences between the sizes of Pah1p expressed in
*S. cerevisiae* and *E. coli* might be explained by posttranslational modifi-
cations of the protein. This notion is supported by the observation that
phosphorylation of Pah1p in *S. cerevisiae* results in a mobility shift to a
position of higher molecular mass in SDS-polyacrylamide gels (56).
The PAH1-encoded Mg\(^{2+}\)-dependent PA phosphatase contains an HAD-like domain with a catalytic DDXDT motif found in a superfamily of Mg\(^{2+}\)-dependent phosphatase enzymes (51, 52). In contrast, the DPP1- and LPP1-encoded Mg\(^{2+}\)-independent PA phosphatases contain a catalytic motif consisting of the consensus sequences KXXXXXRY (domain 1), PSGH (domain 2), SRXXXXXXHXXD (domain 3), which is shared by a superfamily of lipid phosphatases that do not require Mg\(^{2+}\) ions for activity (70–72). Distinctive phosphatase motifs found in the different types of PA phosphatase provide an explanation as to why attempts to identify a Mg\(^{2+}\)-dependent PA phosphatase gene by sequence homology to a Mg\(^{2+}\)-independent PA phosphatase have been unsuccessful. Thus, while both forms of PA phosphatase act to catalyze the same overall reaction, it is expected that their catalytic mechanisms would be different. Another major difference between the two types of PA phosphatase enzymes is the nature in which they associate with membranes. The DPP1- and LPP1-encoded Mg\(^{2+}\)-independent PA phosphatases are integral membrane proteins with six transmembrane-spanning regions (14, 15). On the other hand, the PAH1-encoded Mg\(^{2+}\)-independent PA phosphatase does not have any transmembrane-spanning regions. This enzyme was associated with both the membrane and cytosolic fractions of the cell, and the membrane-bound enzyme was a peripheral membrane protein.

Mg\(^{2+}\)-dependent and -independent PA phosphatase enzymes have been classified as being NEM-sensitive and NEM-insensitive, respectively (10, 12, 73, 74). However, sensitivity to NEM is not an appropriate criterion to classify the two types of PA phosphatase enzymes because each type contains both NEM-sensitive and NEM-insensitive enzymes. For example, the DPP1-encoded phosphatase is NEM-insensitive (19), whereas the LPP1-encoded phosphatase is NEM-sensitive (20). Likewise, the PAH1-encoded Mg\(^{2+}\)-independent PA phosphatase activity was insensitive to NEM, whereas most of the Mg\(^{2+}\)-dependent PA phosphatase activity remaining in the pah1Δ dpp1Δ lpp1Δ triple mutant was sensitive to NEM.

Pah1p (Smp2p) has been recently identified as a factor that coordinates phospholipid synthesis with nuclear/ER membrane growth (56). This conclusion is based on the correlation between massive nuclear/ER membrane expansion and the derepression of the phospholipid synthesis genes (56). Chromatin immunoprecipitation analysis indicates that Pah1p (Smp2p) has been recently identified as a factor that coordinates phospholipid synthesis with nuclear/ER membrane expansion (56). In a mouse model, lipin 1 deficiency prevents normal adipose tissue development that results in lipodystrophy and insulin resistance, whereas excess lipin 1 promotes obesity and insulin sensitivity (30, 65). Despite the importance of lipin 1, the mechanism by which it affects lipodystrophy and obesity has been an enigma due to the lack of information on the molecular function of the protein. In this work, we found that human lipin 1 is a Mg\(^{2+}\)-dependent PA phosphatase, the penultimate enzyme in the pathway to synthesize TAG. This finding provides a mechanistic basis for how lipin 1 regulates lipid metabolism in mammalian cells. Moreover, this work indicated that Mg\(^{2+}\)-dependent PA phosphatase activity might be an important pharmacological target to control lipid metabolism in humans.

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