The Saccharomyces cerevisiae PAH1-encoded Mg$^{2+}$-dependent phosphatidate phosphatase (PAP1, 3-sn-phosphatidate phosphohydrolase, EC 3.1.3.4) catalyzes the dephosphorylation of phosphatidate to yield diacylglycerol and Pi. This enzyme plays a major role in the synthesis of triacylglycerols and phospholipids in S. cerevisiae. PAP1 contains the DXDX(T/V) catalytic motif (DIDGT at residues 398–402) that is shared by the mammalian fat-regulating protein lipin 1 and the superfamily of haloacid dehalogenase-like proteins. The yeast enzyme also contains a conserved glycine residue (Gly$^{80}$) that is essential for the fat-regulating function of lipin 1 in a mouse model. In this study, we examined the roles of the putative catalytic motif and the conserved glycine for PAP1 activity by a mutational analysis. The PAP1 activities of the D398E and D400E mutant enzymes were reduced by >99.9%, and the activity of the G80R mutant enzyme was reduced by 98%. The mutant PAH1 alleles whose products lacked PAP1 activity were nonfunctional in vivo and failed to complement the pah1Δ mutant phenotypes of temperature sensitivity, respiratory deficiency, nuclear/endoplasmic reticulum membrane expansion, derepression of INO1 expression, and alterations in lipid composition. These results demonstrated that the PAP1 activity of the PAH1 gene product is essential for its roles in lipid metabolism and cell physiology.

PAH1 is a gene whose mutation results in increased plasmid maintenance and causes slow growth, temperature sensitivity, and respiratory deficiency. Indeed, Pah1p is the yeast homolog of the mammalian fat-regulating protein lipin 1 (12) and a key regulator of phospholipid biosynthetic gene transcription and nuclear/ER membrane growth (9). Indeed, pah1Δ mutants exhibit derepressed levels of INO1 (encoding inositol-3-phosphate synthase) and OPI3 (encoding phospholipid methyltransferase) (Fig. 1) and massive expansion of the nuclear/ER membrane (9). These observations indicate that Pah1p plays a role as a transcriptional repressor of membrane phospholipid synthesis (9). Moreover, its mammalian counterpart lipin 1 exhibits PAP1 activity (1, 13) and is also characterized as a transcriptional regulator of lipid metabolism (14). Thus, yeast Pah1p and mammalian lipin 1 function as enzymes and transcriptional regulators.

Pah1p has a DXDX(T/V) (DIDGT at residues 398–402) catalytic motif within a haloacid dehalogenase-like domain (Fig. 2). This motif is found in mammalian lipin 1 and the superfamily of Mg$^{2+}$-dependent phosphatase enzymes (15, 16). In this study, we carried out a mutational analysis of the DXDX(T/V) catalytic motif in Pah1p and demonstrated that the conserved aspartate residues in the motif were required for the catalytic function of the protein. We also demonstrated that the conserved glycine (residue 80) within the NLIP domain of Pah1p (Fig. 2) that is essential for the fat-regulating function of lipin 1 in a mouse model (12) was required for the PAP1 activity of the protein. Furthermore, we used catalytic site mutants to demon-
strate that phenotypes (e.g. derepression of INO1 gene expression) associated with the pah1Δ mutation were specifically because of the loss of PAP1 activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Growth medium components were purchased from Difco. Restriction endonucleases, modifying enzymes, and Vent DNA polymerase were purchased from New England Biolabs. Plasmid isolation and gel extraction kits and Ni²⁺-nitrilotriacetic acid-agarose resin were purchased from Qiagen. Biotols. Plasmid isolation and gel extraction kits and Ni²⁺-nitrilotriacetic acid-agarose resin were purchased from Qiagen. Nucleotides, isopropyl β-D-thiogalactoside, phenylmethylsulfonyl fluoride, benzamide, aprotinin, leupeptin, pepstatin, and Triton X-100 were purchased from Sigma. Protein assay reagents, electrophoretic reagents, and protein size standards were purchased from Bio-Rad. Mouse monoclonal antibody assay reagents, electrophoretic reagents, and protein size standards were purchased from Pierce. Polyvinylidene difluoride membranes and the enhanced chemiluminescence Western blotting reagent were purchased from GE Healthcare. Scintillation counting supplies were purchased from National Diagnostics. Lipids were purchased from Avanti Polar Lipids.

**Strains and Growth Conditions**—The bacterial and yeast strains used in this work are listed in Table 1. Yeast cells were grown at 30 °C in YEPD medium (1% yeast extract, 2% peptone, 2% glucose), synthetic complete (SC) medium, or complete synthetic medium (17, 18). Complete synthetic medium, which does not contain inositol (18), was used to examine the inositol-mediated expression of the P<sup>INO1</sup>-lacZ reporter gene. Yeast transformation was performed according to the lithium acetate procedure (19). Plasmid-bearing yeast cells were selected in synthetic medium lacking appropriate amino acids. Temperature sensitivity of yeast cells was determined by the lack of growth at 37 °C. Glycerol was used as a nonfermentable carbon source at a final concentration of 2% in SC medium. *Escherichia coli* cells were grown at 37 °C in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4). *E. coli* transformation was performed by the method of Hanahan (20). Plasmid-bearing *E. coli* cells were selected in growth medium containing ampicillin (100 μg/ml). *E. coli* strains DH5α and BL21(DE3)pLysS were used for plasmid maintenance and protein expression, respectively. Solid growth media for yeast and *E. coli* cells contained agar at a final concentration of 2 and 1.5%, respectively. Cell density in liquid culture was determined by measuring absorbance at 600 nm.

**DNA Manipulations, PCR, and Site-directed Mutagenesis**—Standard methods were used for isolation and manipulation of DNA (21). PCRs were optimized as described by Innis and Gelfand (22). Site-specific mutations in plasmids were generated using the QuikChange site-directed mutagenesis kit (Stratagene).

**Plasmid Constructions**—The plasmids used in this work are listed in Table 2. Plasmid pH316 was constructed by subcloning of the 2.0-kb (XbaI/BglII) and 1.8-kb (BglII/HindIII) PAH1<sup>HA</sup> DNA fragments, which had been released from pGHS12 (1), into pRS415 at the sites of XbaI and HindIII. Plasmid pH323 was derived from pH359 (23) by replacing *URA3* of the P<sup>INO1</sup>-lacZ reporter plasmid with TRP1. For this construction, pH359 was digested with Sall and Stul to remove the *URA3* sequence. The 1-kb TRP1 DNA was amplified from pRS314 by PCR (forward primer, 5'-CCTGAGAGTGCACCCATAAAC-3'; reverse primer with a Sall site (underlined), 5'-TTAAGTCGACCGCGAAGTG-3'). The TRP1 PCR products were digested with Sall, followed by ligation with the Sall/Stul-digested plasmid to produce pH323. The yeast and *Escherichia coli* expression plasmids containing the mutant PAH1<sup>HA</sup> alleles were produced by PCR-mediated site-directed

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**TABLE 1**

<table>
<thead>
<tr>
<th>Strain</th>
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<tr>
<td>E. coli DH5α</td>
<td>F&lt;sup&gt;-&lt;/sup&gt; Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r&lt;sup&gt;K&lt;/sup&gt; m&lt;sup&gt;L&lt;/sup&gt;&lt;sup&gt;+) supE44 thi-1 gyrA96 relA1 ompT hsdS&lt;sup&gt;2&lt;/sup&gt; (m&lt;sup&gt;B&lt;/sup&gt; +/m&lt;sup&gt;B&lt;/sup&gt; +) gal dcm (DE3) pLysS</td>
<td>21 Novagen</td>
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<tr>
<td>E. coli BL21(DE3)pLysS</td>
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<tr>
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<td>67</td>
</tr>
<tr>
<td>GHY57</td>
<td>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 pah1Δ::URA3</td>
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TABLE 2
Plasmids used in this work

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</tr>
<tr>
<td>pGH313</td>
<td>Multicopy E. coli/yeast shuttle vector with LEU2</td>
<td>This study</td>
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<td>This study</td>
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<td>pGH312-G80R</td>
<td>pGH312 containing the G80R mutation in the PAH1 coding sequence</td>
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<td>pGH313 containing the D398E mutation in the PAH1 coding sequence</td>
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<td>pGH313-G80R</td>
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</table>
| pH359                          | P

Preparation of Cell Extracts and Immunoprecipitation—All steps were performed at 4 °C. Yeast cells were harvested at 1,500 × g for 5 min, washed with water, and resuspended in 50 mM Tris-HCl (pH 7.5) buffer containing 0.3 M sucrose, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethanesulfonfyl fluoride, 1 mM benzamidine, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin. The cells were disrupted with glass beads (0.5 mm diameter) using a Biospec Products Mini-BeadBeater-8 as described previously (24), and cell extracts were separated from unbroken cells and glass beads by centrifugation at 1,500 × g for 10 min. Protein concentration of cell extracts was estimated by the method of Bradford (25) using bovine serum albumin as the standard. For immunoprecipitation of Pah1p69, cell extracts (100 μg of protein) were incubated with gentle rotation for 2 h at 4 °C with 1 μg of mouse monoclonal anti-HA antibodies (12CA5) in 500 μl of 50 mM Tris-Cl buffer (pH 7.5) containing 150 mM NaCl, 1% Triton X-100, 0.5 mM PMSF, 1 mM benzamidine, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin. The reaction mixture was incubated for 1 h with 100 μl of protein A-Sepharose beads (10% slurry), followed by centrifugation at 2,500 × g for 1 min at 4 °C. The immunoprecipitates were used for immunoblot analysis or the measurement of PAP1 activity.

SDS-PAGE and Immunoblot Analysis—Proteins were separated by SDS-PAGE (26) using a 7% slab gel. For immunoblot analysis (27), proteins in the gel were transferred to a polyvinylidene difluoride membrane. The membrane was probed first with mouse monoclonal anti-HA antibodies (12CA5) at a dilution of 1:1000, and then with goat anti-mouse IgG-alkaline phosphatase conjugates at a dilution of 1:5000. After development of the membrane using enhanced chemiluminescence detection reagents, fluorescent signals were detected with a FluorImager. The immunoblot signals were in the linear range of detection.

Preparation of 32P-Labeled PA—[32P]PA was synthesized from DAG and [γ-32P]ATP using E. coli DAG kinase as described by Carman and Lin (28).

Expression and Purification of His6-tagged PAP1 Enzymes—The His6-tagged wild type, D398E, D400E, and G80R mutant PAH1-encoded PAP1 enzymes were expressed and purified from E. coli as described by Han et al. (1).

Enzyme Assays—PA phosphatase activity was measured for 20 min at 30 °C in a total reaction volume of 0.1 ml by following the release of water-soluble 32P, from chloroform-soluble [32P]PA (28). For measurement of PAP1 activity, the reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM [32P]PA (10,000–100,000 cpm/nmol), 2 mM Triton X-100, 1 mM MgCl2, 10 mM 2-mercaptoethanol, and enzyme protein. For the measurement of Mg2+-independent PA phosphatase (PAP2) activity, the same reaction mixture was used except for the substitution of 1 mM EDTA for 1 mM MgCl2. Because PAP2 enzymes are active in the presence of 1 mM MgCl2, the PAP1 activity in cell extracts was calculated by subtracting the PA phosphatase activity measured in the absence of MgCl2. A unit of PA phosphatase activity was defined as the amount of enzyme that catalyzed the dephosphorylation of 1 nmol of PA/min. Specific activity was defined as units/mg of protein. The β-galactosidase activity was measured for 10 min at room temperature by following the release of O-nitrophenol from O-nitrophenyl β-D-galactopyranoside at 410 nm using a Beckman DU640 spectrophotometer. The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.0), 1 mM MgCl2, 100 mM 2-mercaptoethanol, 3 mM O-nitrophenyl-β-D-galactopyranoside, and enzyme in a total volume of 0.1 ml. A unit of β-galactosidase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of O-nitrophenol/min. Specific activity was defined as units/mg of protein. All enzyme assays were conducted in triplicate, and the average standard deviation of the assays was ± 5%. The enzyme reactions were linear with time and protein concentration.

Labeling and Analysis of Lipids—Yeast cells were incubated with [2-14C]acetate and 32P, for steady-state labeling of neutral
The D398E and D400E Mutations Abolish PAH1-encoded PAP1 Activity—The PAH1-encoded PAP1 contains the sequence DIDGT (residues 398–402), which corresponds to the DDXD(T/N) catalytic motif found in the superfamily of haloacid dehalogenase-like proteins (Fig. 2). To determine the catalytic role of the DDXD(T/N) motif, we examined the mutational effects of the motif on PAH1-encoded enzyme activity. By using site-directed mutagenesis, we constructed mutant PAH1 alleles that contained glutamate in place of the conserved aspartate residue (Asp398 or Asp400) in the DIDGT sequence. We chose glutamate to replace the aspartate residues to conserve the charge of the amino acid. For immunological detection, the D398E and D400E alleles contained an HA epitope at the N terminus of the PAH1 coding sequence. The mutant PAH1* enzymes were expressed on a multicopy plasmid and the D398E and D400E PAH1* alleles were expressed in the pah1Δ mutant to obviate any effect from the chromosomal wild type PAH1 allele. Immunoblot analysis using anti-HA antibodies showed that the wild type and mutant PA1 enzymes were expressed at comparable levels.

PAP1 and PAP2 activities were assayed in cell extracts that were prepared from pah1Δ mutant cells harboring the wild type, D398E, and D400E PAH1* alleles. The pah1Δ mutant retains DPP1- (33) and LPP1-encoded (34) PAP2 activities that are active in the presence of Mg2+ ions and another PAP1 activity whose molecular identity is yet unknown (1). Thus, to better assess the enzyme activity encoded by the wild type and mutant PAH1* alleles, we differentiated the two types of PA phosphatase activities by measuring activity in the absence and presence of EDTA. PAP2 activity was not affected by expression of the wild type and mutant PAH1* alleles (Fig. 3A). However, the overexpression of the wild type PAH1* allele in pah1Δ mutant cells resulted in a 6-fold increase in PAP1 activity (Fig. 2A). Unlike the wild type PAH1 allele, overexpression of the D398E and D400E PAH1* alleles did not have a significant effect on the levels of PAP1 activity (Fig. 3A).

Anti-HA antibodies were used to immunoprecipitate the HA-tagged PAH1-encoded wild type and mutant enzymes that were expressed the pah1Δ mutant cells, and the immunoprecipitates were used to measure PAP1 activity. Immunoblot analysis using anti-HA antibodies confirmed the presence of the HA-tagged wild type and mutant enzymes in the immunoprecipitates. The lack of an immunoreactive signal in the immunoprecipitate derived from the pah1Δ mutant bearing the vector control indicated that the immune complex formation was specific for HA-tagged protein. Moreover, the lack of PAP1 activity in the vector control immunoprecipitate showed that the immune complex did not contain any PAP1 or PAP2 activity and another PAP1 activity whose molecular identity is yet unknown (1). Thus, to better assess the enzyme activity encoded by the wild type and mutant PAH1* alleles, we differentiated the two types of PA phosphatase activities by measuring activity in the absence and presence of EDTA. PAP2 activity was not affected by expression of the wild type and mutant PAH1* alleles (Fig. 3A). However, the overexpression of the wild type PAH1* allele in pah1Δ mutant cells resulted in a 6-fold increase in PAP1 activity (Fig. 2A). Unlike the wild type PAH1 allele, overexpression of the D398E and D400E PAH1* alleles did not have a significant effect on the levels of PAP1 activity (Fig. 3A).

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His6-tagged wild type, D398E, and D400E PAH1-encoded PAP1 enzymes were expressed and purified from E. coli. As described previously (1), the wild type PAH1-encoded protein exhibited a high level of PAP1 activity and exhibited cooperative kinetics with respect to the surface concentration of PA (Fig. 4). The PAP1 activity of the D398E and D400E mutant enzymes was <0.1% of the activity of the wild type enzyme. This low level of activity (~0.4 nmol/min/mg) could only be measured when the concentration of the enzyme and the specific activity of [32P]PA were increased by 50- and 10-fold, respectively. We could not determine kinetic constants for D398E and D400E mutant enzymes because their activities were not dependent on PA concentration in a meaningful kinetic man-
Yeast Phosphatidate Phosphatase

FIGURE 3. Effects of the D398E, D400E, and G80R mutations on PAH1-encoded PAP1 activity. A, pah1Δ mutant cells expressing the indicated PAH1HA alleles were grown to the exponential phase of growth. Cell extracts were prepared and assayed for PA phosphatase activity in the presence (PAP1) and absence (PAP2) of MgCl₂, EDTA (1 mM) was included in the reaction mixture for the measurement of activity in the absence of MgCl₂. The expression of the wild type and mutants enzymes was confirmed by immunoblot analysis using anti-HA antibodies. B, cell extracts (100 μg of protein) derived from pah1Δ mutant cells expressing the indicated PAH1HA alleles were incubated with anti-HA antibodies for 2 h. Immune complexes were precipitated with protein A-Sepharose and assayed for PAP1 activity. The amount of PAP1 activity in the immune complexes (%, PAP1 Activity/Protein) was determined by immunoblot analysis using anti-HA antibodies. The PAP1 activity in the immune complexes (~2% of the wild type) derived from cells expressing the vector control, D398E, D400E, and G80R PAH1HA alleles were at the limit of detection for the assay.

FIGURE 4. Effects of the D398E, D400E, and G80R mutations on the dependence of PAP1 activity on the surface concentration of PA. The indicated purified recombinant PAH1-encoded proteins were assayed for PAP1 activity as a function of the surface concentration (mol %) of PA. The molar concentration of PA was held constant at 0.2 mM. The error bars fall within the size of the symbols. The break in the plot is between 30 and 100 nmol/min/mg.

does not express lipin 1 because of gross abnormalities of the LPIN1 gene (12). To examine the importance of Gly80 to the enzymatic function of S. cerevisiae Pah1p, we constructed a mutant PAH1144 allele that contained arginine in place of the conserved glycine residue (i.e. G80R mutation). The G80R mutant protein was expressed in yeast and in E. coli with the same vectors that were used to express and isolate the D398E and D400E mutant proteins. Although the G80R mutation did not affect the expression of Pah1p in yeast (as determined by immunoblot analysis using anti-HA antibodies), the mutation had a major effect on its PAP1 activity. For example, the overexpression of the G80R mutant protein did not have a significant effect on the level of PAP1 activity in pah1Δ mutant cells when compared with cells that overexpressed the wild type PAH1144 allele (Fig. 3A). Moreover, the immune complex containing the G80R mutant protein that was isolated from pah1Δ cells expressing the G80R PAH1144 allele had essentially no PAP1 activity (Fig. 3B).

The His6-tagged G80R mutant protein was expressed and purified from E. coli and then examined for its PAP1 activity. The G80R mutation caused a 98% reduction in PAP1 activity (Fig. 4 and Table 3). The Vₘₐₓ of the G80R mutant enzyme was 56-fold lower than that of the wild type enzyme. The G80R mutation also caused a 2.5-fold increase in the cooperativity (i.e. Hill number) with respect to PA, and thus caused decreased activity at low PA concentrations. The G80R mutation, however, did not have a major effect on the Kₘ value for PA. This would suggest that Gly80 did not have a major effect on substrate binding.

Loss of PAH1-encoded PAP1 Activity Is Responsible for the Temperature Sensitivity and Respiratory Deficiency Phenotypes of the pah1Δ Mutant—pah1Δ mutant cells exhibit changes in cell physiology that are reflected in temperature sensitivity and respiratory deficiency (1, 11). To determine whether these phe-
notiptypic changes result from the absence of PAH1-encoded PAP1 activity or from some other function associated with Pah1p, we examined the growth phenotypes of pah1Δ cells expressing the D398E and D400E PAH1ΔA alleles that are mutated for PAP1 activity (Fig. 5). As described previously (1, 11), the pah1Δ mutant exhibited a growth defect at 37 °C on glucose-containing medium and failed to grow on medium containing the nonfermentable carbon source glycerol (i.e. respiratory deficiency). The expression of the wild type PAH1ΔA allele in the pah1Δ mutant complemented the temperature sensitivity and respiratory deficiency of the pah1Δ mutant. Expression of the D398E and D400E PAH1ΔA alleles, however, did not complement these phenotypes. Moreover, the expression of the G80R PAH1ΔA allele, which was also mutated for PAP1 activity, did not complement the temperature sensitivity and respiratory deficiency phenotypes of the pah1Δ mutant (Fig. 5). These data supported the conclusion that PAH1-encoded PAP1 activity was required for normal nuclear/ER membrane structure.

Loss of PAH1-encoded PAP1 Activity Is Responsible for Aberrant Regulation of INO1 Expression—pah1Δ mutant cells exhibit derepressed levels of the phospholipid biosynthetic genes INO1 and OPI3 (9). The expression of these genes is coordinately regulated by nutrient supplementation (e.g. inositol) through a common UASINO element in their promoters (35, 36). In this study, INO1 was used as a representative UASINO-containing gene to examine regulation by PAH1. The effects of the D398E and D400E mutations in PAH1-encoded PAP1 on the expression of INO1 were examined using the PINO1-lacZ reporter gene. The β-galactosidase activity directed by the $P_{INO1}$-lacZ reporter gene was 2.7-fold greater in pah1Δ mutant cells when compared with pah1Δ cells that expressed the wild type PAH1ΔA allele (Fig. 7). Thus, as described previously (9), the deletion of the PAH1 gene resulted in the derepression of INO1. The expression of the D398E and D400E mutant PAH1ΔA alleles, however, did not complement the derepressed level of INO1 expression in the pah1Δ mutant (Fig. 7).

INO1, as well as several other UASINO-containing genes (Fig. 1), is repressed by the supplementation of inositol to the growth medium (35, 36). The $P_{INO1}$-lacZ-directed β-galactosidase activity

### TABLE 3
Effects of mutations on the kinetic constants of PAH1-encoded PAP1 activity

<table>
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<th>Mutant</th>
<th>Vmax (nmol/min/mg)</th>
<th>Km (mol %)</th>
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<td>2.5</td>
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<td>D398E</td>
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<td>NDa</td>
<td>ND</td>
</tr>
<tr>
<td>D400E</td>
<td>0.36a</td>
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<td>G80R</td>
<td>28.6</td>
<td>4.9</td>
<td>6.4</td>
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</tbody>
</table>

*a The enzyme did not follow a meaningful kinetic pattern. The value shown in the table was the highest activity observed.

*a ND, not determined.
activity in pah1Δ mutant cells expressing the wild type PAH1H4A allele was reduced by 30-fold by the addition of inositol to the growth medium (Fig. 7). The expression of INO1 was also reduced in pah1Δ mutant cells, but only by 6-fold (Fig. 7). Moreover, the expression of INO1 in inositol-supplemented pah1Δ mutant cells was 12-fold greater when compared with inositol-supplemented pah1Δ mutant cells expressing the wild type PAH1H4A allele. These results indicated that PAH1 played a role in the inositol-mediated regulation of INO1 expression. The derepressed levels of INO1 expression observed in the pah1Δ mutant grown in the presence of inositol were also observed in pah1Δ mutant cells that expressed the D398E and D400E mutant PAH1H4A alleles (Fig. 7). Thus, the loss of the PAP1 activity of Pah1p was responsible for aberrant regulation of INO1 expression.

Loss of PAH1-encoded PAP1 Activity Is Responsible for aberrant Regulation of Lipid Composition—Previous studies have shown that the deletion of the PAH1 gene results in decreased levels of DAG and TAG (1). These changes are most pronounced in the stationary phase of growth (1) where TAG synthesis predominates over phospholipid synthesis (37, 38). Because the product of the PAP1 reaction (i.e. DAG) is directly used for the synthesis of TAG (39, 40), the reductions in DAG and TAG have been ascribed to the lack of the PAH1-encoded PAP1 enzyme (1). In addition, the pah1Δ mutation causes increased levels of ergosterol esters and free fatty acids (1). To determine whether the changes in neutral lipid composition were specifically because of the lack of PAH1-encoded PAP1 activity, lipid composition was examined in stationary phase pah1Δ mutant cells expressing the D398E and D400E mutant PAH1H4A alleles (Fig. 8). The effects of the pah1Δ mutation on neutral lipid composition were complemented by the expression of the wild type PAH1H4A allele. However, the expression of the D398E and D400E mutant PAH1H4A alleles in the pah1Δ mutant did not complement the effects of the pah1Δ mutation on neutral lipid composition.

The pah1Δ mutation affects phospholipid composition, especially in the exponential phase of growth (1). For example, pah1Δ mutant cells exhibit increased levels of PE, PI, and PA, and a decreased level of PC (1). The specific role of PAH1-encoded PAP1 activity in the regulation of phospholipid composition was examined in exponential phase pah1Δ mutant cells expressing the wild type and mutant PAH1H4A alleles (Fig. 9). The alterations in phospholipid composition caused by the pah1Δ mutation were complemented by the expression of the wild type PAH1H4A allele. The expression of the D398E and D400E mutant PAH1H4A alleles, however, did not complement the alterations in phospholipid composition. Thus, the alter-
DISCUSSION

S. cerevisiae Pah1p has been characterized as a PAP1 enzyme that catalyzes the penultimate step in TAG synthesis (1) and as a transcriptional regulator of phospholipid synthesis and nuclear/ER membrane growth (9). The functions ascribed to Pah1p are based on phenotypes associated with the loss of Pah1p because of a null allele mutation of the PAH1 gene (1, 9, 11). A major aim of this work was to determine whether the phenotypes associated with the pah1Δ mutation were specifically because of the loss of PAP1 activity or because of the loss of another function associated with the protein. To address this question, we constructed and analyzed PAH1 alleles that have mutations in the DXDX(T/V) catalytic motif found in the deduced primary structure of Pah1p. Whether the D398E and D400E mutant proteins were expressed in yeast or in E. coli, they lacked PAP1 activity. Thus, the conserved aspartate residues in the DXDX(T/V) motif were essential for the catalytic function of Pah1p. Moreover, the expression of catalytically inactive Pah1p failed to complement phenotypes associated with the pah1Δ mutation, including aberrant nuclear/ER membrane expansion and regulation of phospholipid synthesis gene expression.

The effect of the G80R mutation on the PAP1 activity of Pah1p was also examined. This is a conserved mutation that is found in the fldΔ mouse that exhibits lipodystrophy and fatty liver at birth (12). The G80R mutation resulted in a 98% reduction in PAP1 activity. The reduction in activity was primarily because of a decrease in catalytic efficiency as reflected in the much reduced Vmax value for the enzyme. The mutation also caused an increase in the cooperative behavior of the enzyme with respect to PA. However, the mutation did not have a major affect on the Km value. Interestingly, the G80R mutation had a major effect on the catalytic step of the PAP1 reaction despite the fact that Gly80 is distant from the DXDX(T/V) catalytic motif in the primary structure of Pah1p (Fig. 2). We speculate that Gly80 might be in close proximity to the DIDGT catalytic sequence of the native (folded) enzyme and that it participates in catalysis. A structural analysis of the enzyme will be needed to address this hypothesis.

The DXDX(T/V) catalytic motif of PAH1-encoded PAP1 differs from the catalytic motif responsible for the PAP2 activities encoded by the S. cerevisiae DPP1 (33, 41) and LPP1 (34) genes. The DPP1- and LPP1-encoded PAP2 enzymes contain a three-domain catalytic motif with consensus sequences KXXXXXRP (domain 1), PSGH (domain 2), and SRXXXXXXHD (domain 3) (33, 34). This motif is shared by a superfamily of lipid phosphatases that do not require Mg2+ ions for activity (42–44). Thus, although the PAP1 and PAP2 enzymes catalyze the same reaction (i.e. dephosphorylation of PA), they do so by different reaction mechanisms. The PAP1 and PAP2 enzymes of S. cerevisiae are also differentiated by their substrate specificity and by the nature in which they associate with membranes. PAH1-encoded PAP1 is specific for PA (1, 2), whereas the DPP1- and LPP1-encoded enzymes utilize a variety of lipid phosphate substrates that include PA, lyso-PA, diacylglycerol pyrophosphate, sphingoid base phosphates, and isoprenoid phosphates (33, 34, 45–47). The DPP1- and LPP1-encoded enzymes are integral membrane proteins that are associated with the vacuole (33, 48, 49) and Golgi (34, 50) compartments, respectively, whereas PAH1-encoded PAP1 is a cytosolic and peripheral membrane protein (1). The DPP1- and LPP1-encoded PAP2 enzymes are postulated to play a role in lipid signaling by generating and/or attenuating the bioactive functions of their substrates and products (3, 51).

The phenotypes (i.e. temperature sensitivity, respiratory deficiency, and aberrant nuclear/ER membrane expansion) that have been described for pah1Δ mutants indicate important roles of PAH1 in cell physiology (9, 11). The lack of complementation by the D398E and D400E mutant PAH1ΔA alleles demonstrated that the specific loss of PAH1-encoded PAP1 activity is the molecular basis for the pah1Δ mutant phenotypes of temperature sensitivity and respiratory deficiency. A defect in mitochondrial function may give rise to respiratory deficiency, and mitochondrial function is dependent on the minor membrane phospholipid cardiolipin (52). Cardiolipin accounted for ~1% of the total phospholipids in our phospholipid composition analysis. However, the amount of this phospholipid was not affected by the pah1Δ mutation. Why the defect in PAP1 activity gives rise to respiratory deficiency was not obvious, but it may be related to signaling functions ascribed to PA (53–55), which may lead to alternations in the expression or function of mitochondrial enzymes.

The aberrant nuclear/ER membrane expansion phenotype has been ascribed to an abnormal increase in phospholipid synthesis (9). This conclusion has been based on the fact that membrane expansion requires phospholipid synthesis and that key phospholipid biosynthetic genes (i.e. INO1 and OPI3) are derepressed in pah1Δ mutant cells (9). More recent studies have shown, however, that elevated levels of phospholipid biosynthetic gene expression alone are not sufficient for nuclear/ER membrane expansion (10), suggesting a specific role of Pah1p in the maintenance of nuclear/ER membrane structure. The studies reported here showed that a loss in PAP1 catalytic function was sufficient for aberrant nuclear/ER membrane expansion. The basis for this phenotype may be related to the effects that an elevated level of PA and/or a reduced level of DAG have on the structure and dynamics of the membrane (56–58).

The work presented here using the D398E and D400E mutants demonstrated that the loss of PAH1-encoded PAP1 activity, and not the loss of Pah1p as a transcription factor, was the molecular basis for the derepression of INO1 expression in both the absence and presence of inositol. The role of PAH1-encoded PAP1 in the transcriptional regulation of INO1 (and OPI3) expression may be explained by a model involving the role of PA in the regulation of Opi1p localization. Opi1p is a PA-binding protein (59) that plays a pivotal role in the regulation of UASINO-containing phospholipid biosynthetic genes (5, 35, 36, 60). Based on this model, Opi1p is tethered to the nuclear/ER membrane through its interaction with PA and Scs2p (59, 61). When the level of PA is reduced, Opi1p is released from the nuclear/ER membrane and translocates into the...
nucleus where it represses transcription of INO1 by binding to the transcriptional activator Ino2p. Thus, conditions that increase the levels of PA, such as the decrease in PAP1 activity, inhibit the translocation and repressor function of Opi1p. On the other hand, conditions that decrease the levels of PA, such as the overexpression of PAP1 activity, promote the translocation and repressor function of Opi1p.

INO1 (and other UASINO-containing genes) is maximally expressed when cells are grown in the absence of inositol and repressed when inositol is supplemented to the growth medium (5, 35, 36, 60). The effect of inositol supplementation on phospholipid synthesis gene expression in relation to controlling the levels of PA may be explained by two mechanisms. Upon inositol supplementation, cells synthesize an elevated level of PI through increased substrate availability (62) and draw upon the pool of PA at the nuclear/ER membrane (59). Inositol supplementation also results in an increase in PAP1 activity (63), which contributes to the decrease in the PA pool. The involvement of PAP1 activity in the Opi1p-mediated regulation of phospholipid biosynthetic gene expression is further supported by the observation that the opiiΔ mutation complements the inositol auxotrophy (i.e. because of the repression of INO1) of cells that overexpress activated phosphorylation-deficient PAP1 (10).

The simultaneous deletion of PAH1 and OPI1 shows a synergistic effect on the derepression of INO1 and OPI3 (10). This observation suggests that the Pah1p-mediated regulation of phospholipid biosynthetic gene expression might also occur by an Opi1p-independent mechanism (10). Furthermore, chromatin immunoprecipitation studies have shown that Pah1p associates with the promoters of INO1 and OPI3 in a phosphorylation-dependent manner (9). This observation has led to the suggestion that Pah1p might regulate more directly transcription of these genes (9). Because Pah1p does not contain any DNA-binding motifs, its role in gene expression could be mediated by other factors. Whether the association of Pah1p with chromatin contributes to the transcriptional regulation of phospholipid biosynthetic genes and whether this association requires PAP1 activity requires further investigation.

The DXDX(T/V) catalytic motif within the halocid dehalogenase-like domain and the conserved glycine within the NLIP domain of Pah1p are also found in the three mammalian lipin proteins (51). In mice, loss of lipin 1 prevents normal adipose tissue development resulting in lipodystrophy and insulin resistance, whereas an excess of lipin 1 promotes obesity and insulin sensitivity (12, 64). The homologies between yeast Pah1p and the mammalian lipins, and the discovery that PAH1 encodes a PAP1 enzyme led to the revelation that lipins 1–3 exhibit PAP1 activity (1, 13). That lipin 1 has PAP1 activity provides a mechanistic basis for why the absence of lipin 1 (i.e. fld mouse) and the G84R mutation (i.e. fldG84R mouse) has such a major effect on fat metabolism in mammalian cells (1, 12). Indeed, the G84R mutation in lipin 1 causes an 80% reduction in PAP1 activity when expressed in mammalian 293T cells (65). However, the explanation that the phenotypes exhibited by the fld and fldG84R mice are because of the loss of PAP1 activity alone may be an oversimplification of the complex physiology that occurs in these animals.

Like yeast Pah1p (9), mammalian lipin 1 plays a transcriptional role in the regulation of lipid metabolism (14). This function appears to be more complex than that of Pah1p in yeast. Lipin 1 activates mitochondrial fatty acid oxidative metabolism in liver by inducing the expression of the nuclear receptor PPARα, a target of PPARγ coactivator 1α through interaction with PPARα and PPARγ coactivator 1α (14). However, the transcriptional coactivation function of lipin 1 in liver appears to be independent of its PAP1 activity (14). Lipin 1 expression is also required for adipocyte differentiation and for the expression of lipogenic genes (66). Whether these functions are mediated through physical interaction with other factors and/or require its PAP1 activity warrants further investigation.

REFERENCES
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