The Cellular Functions of the Yeast Lipin Homolog Pah1p Are Dependent on Its Phosphatidate Phosphatase Activity*

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The Saccharomyces cerevisiae PAH1-encoded Mg2+-dependent phosphatidate phosphatase (PAP1, 3-sn-phosphatidate phosphohydrolase, EC 3.1.3.4) catalyzes the dephosphorylation of phosphatidate to yield diacylglycerol and P1. 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 (Gly80) 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 (11). 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 Mg2+-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) 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. Nucleotides, isopropyl β-D-thiogalactoside, phenylmethylsulfonyl fluoride, benzamidine, aprotinin, leupeptin, pepstatin, and Triton X-100 were purchased from Sigma. Protamine sulfate were purchased from Pierce. Polyvinylidene difluoride 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<sub>INO1</sub>-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 pGH316 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 pGH312 (1), into pRS415 at the sites of XbaI and HindIII. Plasmid pGH323 was derived from pH359 (23) by replacing *URA3* of the P<sub>INO1</sub>-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′-CCTGAGAGTCGACGCTAAAC-3′; reverse primer with a Sall site (underlined), 5′-TTATGGCGAAGCGGCAAGTG-3′). The TRP1 PCR products were digested with Sall, followed by ligation with the Sall/Stul-digested plasmid to produce pGH323. 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**

| Strains used in this work | Source or Ref. |
|--------------------------|---------------|
| E. coli                  |               |
| DH5α                     | F– Δ80lacZ1ΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r<sup>K</sup>– m<sup>K</sup>–) phoA supE44 thi-1 gyrA96 relA1 | 21 |
| BL21(DE3)pLysS           | F–ompT hsdS<sup>R</sup> (r<sup>K</sup>– m<sup>K</sup>–) gal dcm (DE3) pLysS | Novagen |
| S. cerevisiae            |               |
| W303-1A                  | MATα ade2–1 can1–100 his3–11,15 leu2–3,112 trp1–1 ura3–1 | 67 |
| GHY57                    | MATα ade2–1 can1–100 his3–11,15 leu2–3,112 trp1–1 ura3–1 pah1Δ::URA3 | 1 |

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**FIGURE 1.** The role of *PAH1*-encoded PAP1 in lipid synthesis. The product of the *PAH1*-encoded PAP1 reaction DAG is used for the synthesis of TAG and for the synthesis of PE and PC via the Kennedy pathway. The enzyme substrate PA is used for the synthesis of all phospholipids and PI-derived sphingolipids via the intermediate CDP-DAG via the CDP-DAG pathway. The UAS<sub>PAH1</sub>-containing genes that are subject to inositol-mediated regulation are indicated in dark gray. Abbreviations used are as follows: PS, phosphatidylserine; PIPs, phosphatidylinositol phosphates; PGP, phosphatidyglycerophosphate; CL, cardiolipin; Glc-6-P, glucose 6-phosphate; Ins-3-P, inositol 3-phosphate; Ins, inositol; Etn, ethanolamine; P-Etn, phosphoethanolamine; CDP-Etn, CDP-ethanolamine; Cho, choline; P-Chol, phosphocholine; CDP-Chol, CDP-choline.
mutagenesis of pGH312 (1) and pGH313 (1), respectively. The codon changes GAT→GAA and GGA→AGA were used for the D398E and D400E, and G80R mutations, respectively. The nucleotide change in the PAH1^{14A} alleles was confirmed by DNA sequencing. Plasmid YCplac22-SEC63-GFP was constructed by subcloning the SEC63-GFP insert, which was released from the YCplac111-SEC63-GFP (10), into YCplac22 at the sites of SacI and HindIII. Plasmid pRS313-RFP-PLUS1 was constructed by subcloning the RFP-PLUS1 insert, which was released from the YCplac33-RFP-PLUS1(10), into pRS313 at the sites of SacI and SalI.

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 mM 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. 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 using bovine serum albumin as the standard. For immunoprecipitation of Pah1p^{14A}, 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-HCl 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
lipids and phospholipids, respectively, as described previously (1). Lipids were extracted from labeled cells by the method of Bligh and Dyer (29). Neutral lipids were separated by TLC on silica gel plates using the solvent system of hexane/diethyl ether/glacial acetic acid (40:10:1, v/v) (30). Phospholipids were separated by two-dimensional TLC on silica gel plates using the solvents of chloroform/methanol/ammonium hydroxide/water (45:25:2:3, v/v) and chloroform/methanol/glacial acetic acid/water (32:4:5:1, v/v) (31). Radiolabeled lipids were visualized by PhosphorImaging analysis, and their identities were confirmed by comparison with lipid standards after exposure to iodine vapor. The relative quantities of labeled lipids were analyzed using ImageQuant software.

Fluorescence Microscopy—The expression of SEC63-GFP and RFP-PUS1 fusion genes were used to visualize the nuclear/ER membrane and nuclear morphology, respectively (10). The pah1Δ mutant bearing the wild type, D398E, and D400E mutant PAH1HA alleles was transformed with the reporter plasmids, and the resulting transformants were selected in SC medium lacking histidine, leucine, and tryptophan. These cells were grown to exponential phase at 30 °C and then examined with a Zeiss Axiovert 200M inverted microscope (63× Plan Apochromat objective) attached with an LSM 510 confocal laser scanning system. For each strain, two independent transformants were examined, and in all cases the results were same for both.

Data Analyses—Kinetic data were analyzed according to the Hill equation using the EZ-FIT enzyme kinetic model-fitting program (32). The Student’s t test (SigmaPlot software) was used to determine statistical significance, and p values < 0.05 were taken as a significant difference.

RESULTS

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 DXDX(T/V) catalytic motif found in the superfamily of haloacid dehalogenase-like proteins (Fig. 2). To determine the catalytic role of the DXDX(T/V) 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 PAH1HA alleles carried on a multicopy plasmid 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 PAH1 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 PAH1HA 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 PAH1HA 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 PAH1HA alleles (Fig. 3A). However, the overexpression of the wild type PAH1HA 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 PAH1HA 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 enzymes (Fig. 3B). The immune complex derived from pah1Δ mutant cells expressing the wild type PAH1HA allele contained PAP1 activity (Fig. 3B). On the other hand, the immune complexes derived from cells expressing the D398E and D400E mutant enzymes had essentially no PAP1 activity (Fig. 3B).

His6-tagged wild type, D398E, and D400E PAH1-encoded PAH1 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 PAH1<sup>HA</sup> 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 MgCl<sub>2</sub>, EDTA (1 mM) was included in the reaction mixture for the measurement of activity in the absence of MgCl<sub>2</sub>. 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 PAH1<sup>HA</sup> 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 (27 pmol/min) in cells expressing the wild type enzyme was set at 100%. The data were normalized to the amounts of immunoprecipitated PAP1 proteins as 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 PAH1 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 μM. The amount of enzyme used for the assay of the wild type and mutant enzymes was 0.1 and 5 μg, respectively. The specific activity of the [32P]PA used for the assay of the wild type and mutant enzymes was 10,000 and 100,000 cpm/nmol, respectively. The data shown were determined from triplicate determinations ± S.D. The error bars fall within the size of the symbols. The break in the plot is between 30 and 100 nmol/min/mg.

The G80R Mutation Causes Massive Reduction in PAH1-encoded PAP1 Activity—Gly<sup>80</sup> that is found in the NLIP domain of Pah1p (Fig. 2) is conserved in mammalian lipin proteins (12). A spontaneous G84R mutation in lipin 1 causes lipodystrophy and fatty liver in the fld<sup>−/−</sup> (fatty liver dystrophy) mouse at birth (12). These phenotypes are also exhibited by the fld mouse that does not express lipin 1 because of gross abnormalities of the LPIN1 gene (12). To examine the importance of Gly<sup>80</sup> to the enzymatic function of S. cerevisiae Pah1p, we constructed a mutant PAH1<sup>HA</sup> 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 PAH1<sup>HA</sup> allele (Fig. 3A). Moreover, the immune complex containing the G80R mutant protein that was isolated from pah1Δ cells expressing the G80R PAH1<sup>HA</sup> allele had essentially no PAP1 activity (Fig. 3B).

The His<sub>6</sub>-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 K<sub>m</sub> 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 affect on the K<sub>m</sub> value for PA. This would suggest that Gly<sup>80</sup> 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-
The expression of the wild type PAP1 activity, did not complement the temperature sensitivity of the pah1 mutant cells expressing the indicated PAH1HA alleles were grown to saturation at 30 °C. After adjustment of the culture to the density of 2 × 10⁷ cells/ml followed by 10-fold serial dilutions to 2 × 10⁴ cells/ml, 10 μl of each diluted culture was spotted onto plates containing 2% glucose or 2% glycerol. The cells on glucose medium were incubated for 3 days at 30 and at 37 °C, respectively, whereas the cells on glycerol medium were incubated for 7 days at 30 °C.

FIGURE 5. Temperature sensitivity and respiratory deficiency of pah1Δ cells bearing the wild type, D398E, and D400E mutant PAH1HA alleles. pah1Δ mutant cells expressing the indicated PAH1HA alleles were grown to saturation at 30 °C. After adjustment of the culture to the density of 2 × 10⁷ cells/ml followed by 10-fold serial dilutions to 2 × 10⁴ cells/ml, 10 μl of each diluted culture was spotted onto plates containing 2% glucose or 2% glycerol. The cells on glucose medium were incubated for 3 days at 30 and at 37 °C, respectively, whereas the cells on glycerol medium were incubated for 7 days at 30 °C.

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

| Mutant     | Vₘₐₓ  | Kₘ   | Hill number |
|------------|-------|------|-------------|
| Wild type  | 1613  | 3.3  | ND          |
| D398E      | 0.40* | ND*  | ND          |
| D400E      | 0.36* | ND*  | ND          |
| G80R       | 28.6  | 4.9  | 6.4         |

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

ND, not determined.

FIGURE 6. Effects of the D398E and D400E mutations on nuclear/ER membrane growth. pah1Δ mutant cells expressing the indicated PAH1HA alleles and bearing plasmids YCplac22-SEC63-GFP (to label the nuclear/ER membrane) and pRS313-RFP-PUS1 (to label the nucleus) were grown to the exponential phase of growth. Fluorescence signals from the reporter proteins were examined using a Zeiss Axiovert 20M inverted microscope attached with an LSM 510 confocal laser scanning system. The white bar indicates 5 μm.

and nucleoplasm, respectively (10). As shown in Fig. 6, pah1Δ cells expressing the wild type PAH1HA allele had normal round-shaped nuclei, indicating that wild type PAH1HA allele complemented the nuclear/ER membrane expansion phenotype of the pah1Δ mutant. By contrast, pah1Δ cells expressing the D398E and D400E mutant PAH1HA alleles had enlarged and irregularly shaped nuclei. 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 PINO1lacZ reporter gene. The β-galactosidase activity directed by the PINO1-lacZ reporter gene was 2.7-fold greater in pah1Δ mutant cells when compared with pah1Δ cells that expressed the wild type PAH1HA 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 PAH1HA 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 PINO1-lacZ-directed β-galactosidase
activity in pah1Δ mutant cells expressing the wild type PAH1HA 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 PAH1HA 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 PAH1HA 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 Ablation 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 PAH1HA alleles (Fig. 8). The effects of the pah1Δ mutation on neutral lipid composition were complemented by the expression of the wild type PAH1HA allele. However, the expression of the D398E and D400E mutant PAH1HA 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 PAH1HA alleles (Fig. 9). The alterations in phospholipid composition caused by the pah1Δ mutation were complemented by the expression of the wild type PAH1HA allele. The expression of the D398E and D400E mutant PAH1HA alleles, however, did not complement the alterations in phospholipid composition. Thus, the alter-

Yeast Phosphatidate Phosphatase

FIGURE 7. Effects of the D398E and D400E mutations on the expression of the P<sup>PAH1</sup>-lacZ reporter gene. pah1Δ mutant cells expressing the indicated PAH1HA alleles and the P<sup>PAH1</sup>-lacZ reporter plasmid pGH323 were grown to exponential phase in the absence and presence of 75 μM inositol. Cell extracts were prepared and assayed for β-galactosidase activity. Each data point represents the average of triplicate enzyme determinations ± S.D.

FIGURE 8. Effects of the D398E and D400E mutations on neutral lipid composition. pah1Δ mutant cells expressing the indicated PAH1HA alleles were grown to the stationary phase in the presence of [2-14C]acetate (1 μCi/ml). Lipids were extracted and separated by one-dimensional TLC, and the phosphorimages were subjected to ImageQuant analysis. The percentages shown for the individual lipids were normalized to the total 14C-labeled chloroform-soluble fraction, which also contained phospholipids and unidentified neutral lipids. Each data point represents the average of three experiments ± S.D. The abbreviations used are as follows: Erg, ergosterol; ErgE, ergosterol ester; FA, fatty acid.

FIGURE 9. Effects of the D398E and D400E mutations on phospholipid composition. pah1Δ mutant cells expressing the indicated PAH1HA alleles were grown to the exponential phase of growth in the presence of [32P]<sub>3</sub>Pi (10 μCi/ml). Phospholipids were extracted and separated by two-dimensional TLC, and the phosphorimages were subjected to ImageQuant analysis. The percentages shown for the individual phospholipids were normalized to the total 32P-labeled chloroform-soluble fraction that included sphingolipids and unidentified phospholipids. Each data point represents the average of three experiments ± S.D. The abbreviation used is as follows: PS, phosphatidylserine.
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 *fld* 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 *V*<sub>max</sub> 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 *K*<sub>m</sub> value. Interestingly, the G80R mutation had a major effect on the catalytic step of the PAP1 reaction despite the fact that Gly<sup>80</sup> is distant from the *DXDX(T/V)* catalytic motif in the primary structure of Pah1p (Fig. 2). We speculate that Gly<sup>80</sup> 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 KXXXKXXXRP (domain 1), PSGH (domain 2), and SRXXXHHXXXD (domain 3) (33, 34). This motif is shared by a superfamily of lipid phosphatases that do not require Mg<sup>2+</sup> 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 proteins of *PAH1*<sup>HA</sup> 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 Opilp localization. Opilp is a PA-binding protein (59) that plays a pivotal role in the regulation of *UAS<sub>INO</sub>*-containing phospholipid biosynthetic genes (5, 35, 36, 60). Based on this model, Opilp is tethered to the nuclear/ER membrane through its interaction with PA and Scs2p (59, 61). When the level of PA is reduced, Opilp 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 op11Δ 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 Phap1p-mediated regulation of phospholipid biosynthetic gene expression might also occur by an Opi1p-independent mechanism (10). Furthermore, chromatin immunoprecipitation studies have shown that Phap1p associates with the promoters of INO1 and OPI3 in a phosphorylation-dependent manner (9). This observation has led to the suggestion that Phap1p might regulate more directly transcription of these genes (9). Because Phap1p does not contain any DNA-binding motifs, its role in gene expression could be mediated by other factors. Whether the association of Phap1p 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 haloacid dehalogenase-like domain and the conserved glycine within the NLIP domain of Phap1p 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 (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 Phap1p 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

1. Han, G.-S., Wu, W.-I., and Carman, G. M. (2006) J. Biol. Chem. 281, 9210–9218
2. Lin, Y.-P., and Carman, G. M. (1989) J. Biol. Chem. 264, 8641–8645
3. Carman, G. M. (1997) Biochim. Biophys. Acta 1348, 45–55
4. Carman, G. M., and Zeimentz, G. M. (1996) J. Biol. Chem. 271, 13293–13296
5. Carman, G. M., and Henry, S. A. (1999) Prog. Lipid Res. 38, 361–399
6. Wu, W.-I., and Carman, G. M. (1996) Biochemistry 35, 3790–3796
7. Wu, W.-I., Lin, Y.-P., Wang, E., Merrill, A. H., Jr., and Carman, G. M. (1993) J. Biol. Chem. 268, 13830–13837
8. Wu, W.-I., and Carman, G. M. (1994) J. Biol. Chem. 269, 29495–29501
9. Santos-Rosa, H., Leung, J., Grimsey, N., Peak-Chew, S., and Siniossoglou, S. (2005) EMBO J. 24, 1931–1941
10. O’Hara, L., Han, G. S., Peak-Chew, S., Grimsey, N., Carman, G. M., and Siniossoglou, S. (2006) J. Biol. Chem. 281, 34357–34358
11. Irie, K., Takase, M., Araki, H., and Oshima, Y. (1993) Mol. Gen. Genet. 236, 285–288
12. Peterfy, M., Phan, J., Xu, P., and Reue, K. (2001) Nat. Genet. 27, 121–124
13. Donkor, I., Sariahmetoglu, M., Dewald, J., Brindley, D. N., and Reue, K. (2007) J. Biol. Chem. 282, 3450–3457
14. Finck, B. N., Gropler, M. C., Chen, Z., Leone, T. C., Croce, M. A., Harris, T. E., Lawrence, J. C., Jr., and Kelly, D. P. (2006) Cell Metab. 4, 199–210
15. Collet, J. F., Stroobant, V., Pirard, M., Delpierre, G., and Van Schaftingen, E. (1998) J. Biol. Chem. 273, 14107–14112
16. Collet, J. F., Stroobant, V., and Van Schaftingen, E. (1999) J. Biol. Chem. 274, 33985–33990
17. Rose, M. D., Winston, F., and Heitler, P. (1990) Methods in Yeast Genetics: A Laboratory Course Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
18. Culbertson, M. R., and Henry, S. A. (1975) Genetics 80, 23–40
19. Ito, H., Yasuki, F., Murata, K., and Kimura, A. (1983) J. Bacteriol. 153, 163–168
20. Hanahan, D. (1983) J. Biol. Chem. 166, 557–580
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
22. Innis, M. A., and Gelfand, D. H. (1990) in PCR Protocols: A Guide to Methods and Applications (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds) pp. 3–12, Academic Press, Inc., San Diego
23. Lopes, J. M., Hirsch, J. P., Chorgo, P. A., Schulze, K. L., and Henry, S. A. (1999) Nucleic Acids Res. 19, 1687–1693
24. Klig, I. S., Homann, M. I., Carman, G. M., and Henry, S. A. (1985) J. Bacteriol. 162, 1135–1141
25. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
26. Laemmli, U. K. (1970) Nature 227, 680–685
27. Haid, A., and Sissa, M. (1983) Methods Enzymol. 96, 192–205
28. Carman, G. M., and Lin, Y.-P. (1991) Methods Enzymol. 197, 548–553
29. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–917
30. Henderson, R. I., and Tocher, D. R. (1992) in *Lipid Analysis* (Hamilton, R. I., and Hamilton, S., eds) pp. 65–111, IRL Press at Oxford University Press, New York
31. Esko, J. D., and Raetz, C. R. H. (1980) *J. Biol. Chem.* 255, 4474–4480
32. Perrella, F. (1988) *Anal. Biochem.* 174, 437–447
33. Toke, D. A., Bennett, W. L., Dillon, D. A., Chen, X., Oshiro, J., Ostrander, D. B., Wu, W.-I., Voelker, D. R., Fischl, A. S., and Carman, G. M. (1998) *J. Biol. Chem.* 273, 3278–3284
34. Toke, D. A., Bennett, W. L., Oshiro, J., Wu, W. I., Voelker, D. R., and Carman, G. M. (1999) *J. Biol. Chem.* 273, 14331–14338
35. Greenberg, M. L., and Lopes, J. M. (1996) *Microbiol. Rev.* 60, 1–20
36. Chen, M., Hancock, L. C., and Lopes, J. M. (2007) *Biochim. Biophys. Acta* 1771, 310–321
37. Taylor, F. R., and Parks, L. W. (1979) *Biochim. Biophys. Acta* 575, 204–214
38. Hosaka, K., and Yamashita, S. (1984) *Biochim. Biophys. Acta* 796, 110–117
39. Sorger, D., and Daum, G. (2003) *Appl. Microbiol. Biotechnol.* 61, 289–299
40. Czabany, T., Athenstaedt, K., and Daum, G. (2007) *Biochim. Biophys. Acta* 1771, 299–309
41. Toke, D. A., McClintick, M. L., and Carman, G. M. (1999) *Biochemistry* 38, 14606–14613
42. Stukey, J., and Carman, G. M. (1997) *Protein Sci.* 6, 469–472
43. Hemrika, W., Renirie, R., Dekker, H. L., Barnett, P., and Wever, R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 2145–2149
44. Neuwald, A. F. (1997) *Protein Sci.* 6, 1764–1767
45. Wu, W.-I., Liu, Y., Riedel, B., Wissing, J. B., Fischl, A. S., and Carman, G. M. (1996) *J. Biol. Chem.* 271, 1868–1876
46. Furneisen, J. M., and Carman, G. M. (2000) *Biochim. Biophys. Acta* 1484, 71–82
47. Faulkner, A. J., Chen, X., Rush, J., Horazdovsky, B., Waechter, C. J., Carman, G. M., and Sternweis, P. C. (1999) *J. Biol. Chem.* 274, 14831–14837
48. Han, G.-S., Johnston, C. N., and Carman, G. M. (2004) *J. Biol. Chem.* 279, 5338–5345
49. Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weisman, J. S., and O’Shea, E. K. (2003) *Nature* 425, 686–691
50. Carman, G. M., and Han, G. S. (2006) *Trends Biochem. Sci.* 31, 694–699
51. Li, G. H., Chen, S., Thompson, M. N., and Greenberg, M. L. (2007) *Biochim. Biophys. Acta* 1771, 432–441
52. English, D., Cui, Y., and Siddiqui, R. A. (1996) *Chem. Phys. Lipids* 80, 117–132
53. Wang, X., Devaiah, S. P., Zhang, W., and Welti, R. (2006) *Prog. Lipid Res.* 45, 250–278
54. Foster, D. A. (2007) *Cancer Res.* 67, 1–4
55. Kooijman, E. E., Chupin, V., de Kruijff, B., and Burger, K. N. (2003) *Traffic* 4, 162–174
56. Shemes, T., Luini, A., Malhotra, V., Burger, K. N., and Kozlov, M. M. (2003) *Biophys. J.* 85, 3813–3827
57. Huijbregts, R. P., Topalof, L., and Bankaitis, V. A. (2000) *Traffic* 1, 195–202
58. Loewen, C. J. R., Gaspar, M. L., Jesch, S. A., Delon, C., Ktistakis, N. T., Henry, S. A., and Levine, T. P. (2004) *Science* 304, 1644–1647
59. Henry, S. A., and Patton-Vogt, J. L. (1998) *Prog. Nucleic Acids Res.* 61, 133–179
60. Loewen, C. J. R., and Levine, T. P. (2005) *J. Biol. Chem.* 280, 14097–14104
61. Kelley, M. J., Bailis, A. M., Henry, S. A., and Carman, G. M. (1988) *J. Biol. Chem.* 263, 18078–18085
62. Morlock, K. R., Lin, Y.-P., and Carman, G. M. (1988) *J. Bacteriol.* 170, 3561–3566
63. Phan, J., and Reue, K. (2005) *Cell Metab.* 1, 73–83
64. Harris, T. E., Huffman, T. A., Chi, A., Shabanowitz, J., Hunt, D. F., Kumar, A., and Lawrence, J. C., Jr. (2007) *J. Biol. Chem.* 282, 277–286
65. Peterfy, M., Phan, J., and Reue, K. (2005) *J. Biol. Chem.* 280, 32883–32889
66. Ostrander, D. B., O’Brien, D. J., Gorman, J. A., and Carman, G. M. (1998) *J. Biol. Chem.* 273, 18992–19001
67. Sikorski, R. S., and Hieter, P. (1989) *Genetics* 122, 19–27
68. Hill, J. E., Myers, A. M., Koerner, T. J., and Tzagoloff, A. (1986) *Yeast* 2, 163–167