Pho85p-Pho80p Phosphorylation of Yeast Pah1p Phosphatidate Phosphatase Regulates Its Activity, Location, Abundance, and Function in Lipid Metabolism*•

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The yeast Pah1p phosphatidate phosphatase, which catalyzes the penultimate step in the synthesis of triacylglycerol and plays a role in the transcriptional regulation of phospholipid synthesis genes, is a cytosolic enzyme that associates with the nuclear/endoplasmic reticulum membrane to catalyze the dephosphorylation of phosphatidate to yield diacylglycerol. Pah1p is phosphorylated on seven (Ser-110, Ser-114, Ser-168, Ser-602, Thr-723, Ser-744, and Ser-748) sites that are targets for proline-directed protein kinases. In this work, we showed that the seven sites are phosphorylated by Pho85p-Pho80p, a protein kinase-cyclin complex known to regulate a variety of cellular processes. The phosphorylation of recombinant Pah1p was time- and dose-dependent and dependent on the concentrations of ATP (3.7 μM) and Pah1p (0.25 μM). Phosphorylation reduced (6-fold) the catalytic efficiency (Vmax/Km) of Pah1p and reduced (3-fold) its interaction (Kd) with liposomes. Alanine mutations of the seven sites ablated the inhibitory effect that Pho85p-Pho80p had on Pah1p activity and on the interaction with liposomes. Analysis of pho85Δ mutant cells, phosphate-starved wild type cells, and cells expressing phosphorylation-deficient forms of Pah1p indicated that loss of Pho85p-Pho80p phosphorylation reduced Pah1p abundance. In contrast, lack of Nem1p-Spo7p, the phosphatase complex that dephosphorylates Pah1p at the nuclear/endoplasmic reticulum membrane, stabilized Pah1p abundance. Although loss of phosphorylation caused a decrease in abundance, a greater amount of Pah1p was associated with membranes when compared with phosphorylated enzyme, and the loss of phosphorylation allowed bypass of the Nem1p-Spo7p requirement for Pah1p function in the synthesis of triacylglycerol.

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2 The abbreviations used are: PAP, phosphatidate phosphatase, PA, phosphatidate; PC, phosphatidylcholine; DAG, diacylglycerol; TAG, triacylglycerol; ER, endoplasmic reticulum; T/V, transverse/vertical.

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show that Pho85p-Pho80p phosphorylation of Pah1p regulates PAP activity, location, abundance, and function in lipid metabolism.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were reagent grade or better. Difco was the supplier of growth medium supplies. Modifying enzymes, recombinant Vent DNA polymerase, and restriction endonucleases were from New England Biolabs. The DNA gel extraction kit, plasmid DNA purification kit, and nickel-nitritolatriacetic acid-agarose resin were purchased from Qiagen. Aprotinin, benzamidine, bovine serum albumin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, phosphoamino acids, l-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, protein A-Sepharose CL-4B, and Triton X-100 were purchased from Sigma-Aldrich. PCR primers were prepared by Genosys Biotechnologies, the QuiKChange site-directed mutagenesis kit was from Stratagene, and carrier DNA for yeast transformation was from Clontech. IgG-Sepharose, polyvinylidene difluoride paper, and the enhanced chemiluminescence Western blotting detection kit were from GE Healthcare. DNA size ladders, electrophoresis reagents, immunochromic reagents, molecular mass protein standards, and protein assay reagents were from Bio-Rad. Lipids and thin-layer chromatography plates (cellulose and silica gel 60) were from Avanti Polar Lipids and EM Science, respectively. Radiochemicals were Perkin-Elmer Life Sciences, and scintillation counting supplies and acrylamide solutions were from National Diagnostics. Alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies, alkaline phosphatase-conjugated goat anti-mouse IgG antibodies, mouse anti-phosphoglycerate kinase antibodies, and mouse anti-(phosphoserine/phosphothreonine)-proline (MPM2) antibodies were from Thermo Scientific, Pierce, Invitrogen, and Millipore, respectively.

**Strains and Growth Conditions**—The strains used in this work are listed in Table 1. *Escherichia coli* strain DH5α was used for the propagation of plasmids. *E. coli* cells were grown at 37 °C in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7). Ampicillin (100 μg/ml) was added to select for cells carrying plasmids. His<sub>SG</sub>-tagged wild type and phosphorylation-deficient forms of Pah1p were expressed in *E. coli* BL21(DE3)pLysS cells bearing the indicated PAH1 derivatives of plasmid pET-15b as described by Han et al. (29). Cells were grown to an A<sub>600</sub> of 0.5 at room temperature in 500 ml of LB medium containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml). Cultures were incubated for 1 h with 1 mm isopropyl β-D-thiogalactoside to induce expression of His<sub>SG</sub>-tagged wild type and mutant forms of Pah1p. Yeast cells were grown in synthetic complete medium. Appropriate amino acids were omitted from the synthetic growth medium to select for cells carrying specific plasmids. High phosphate medium contained 1 mg/ml potassium phosphate, whereas low phosphate medium contained 4.5 μg/ml potassium phosphate plus 1 mg/ml potassium chloride (30). The growth regime described by Komeili and O’Shea (30) was used to deplete phosphorus from cells. Cell numbers in liquid cultures were determined spectrophotometrically at an absorbance of 600 nm. The growth
medium was supplemented with agar (2% for yeast or 1.5% for E. coli) for growth on plates.

DNA Manipulations—Standard protocols were used to isolate genomic and plasmid DNA, digest and ligate DNA, and amplify DNA by PCR (31, 32). Plasmids used in this study are listed in Table 2. Plasmid pGHC131 directs the isopropyl β-D-thiogalactopyranoside-induced overexpression of His6-tagged Pah1p in E. coli (5), whereas plasmid pGHC315 directs low copy expression of Pah1p in yeast (24). The derivatives of pGHC131 and pGHC135 containing PAH1(S110A), PAH1(S114A), PAH1(S168A), PAH1(S602A), PAH1(T723A), PAH1(S744A), and PAH1(S748A) were constructed by QuikChange site-directed mutagenesis using the appropriate mutagenic primers. Plasmids containing combinations of mutations were also constructed. All mutations were confirmed by DNA sequencing. Plasmids EB1164 and EB1076 were used for the overexpression of His6-tagged Pho85p and untagged Pho80p, respectively, in E. coli (33). Plasmid transformations of E. coli (31) and yeast (34) were performed as described previously.

Preparation of Cell Extracts, Subcellular Fractionation, and Purification of Recombinant Enzymes—All steps were performed at 4°C. Cell extracts were prepared by disruption of yeast cells with glass beads (0.5-mm diameter) using a BioSpec Products Mini-BeadBeater-16 (35). The lysis buffer contained 50 mM Tris-HCl, pH 7.5, 0.3 mM sucrose, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin. The cytosol (supernatant) and total membrane (pellet) fractions were separated by centrifugation at 100,000 × g for 1 h (35). The membrane pellets were suspended in the lysis buffer to the same volume as the cytosol fraction. His6-tagged wild type and mutant forms of Pah1p expressed in E. coli BL21(DE3)pLysS were purified by affinity chromatography using nickel-nitrilotriacetic acid-agarose as described by Han et al. (5). SDS-PAGE analyses showed that the purified preparations were nearly homogeneous. His6-tagged Pho85p-Pho80p complex was purified from E. coli BL21(DE3) expressing plasmids EB1164 and EB1076 (33). E. coli BL21(DE3) expressing plasmid EB1164 was used to purify His6-tagged Pho85p. Protein concentration was determined by the method of Bradford (36) using bovine serum albumin as the standard.

Phosphorylation Reactions—Phosphorylation reactions were routinely performed in triplicate for 10 min at 30 °C in a total volume of 20 μl. The standard reaction contained 25 μl Tris-HCl, pH 7.5, 10 mM MgCl2, 2 mM dithiothreitol, 100 μM [γ-32P]ATP (3,000 cpm/pmol), 1 μg of Pah1p, and the indicated amounts of Pho85p-Pho80p. At the end of the phosphorylation reactions, samples were treated with 4× Laemmli buffer (37), subjected to SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Phosphorimaging was used to visualize phosphorylated enzyme, and the extent of phosphorylation was quantified with ImageQuant software. A unit of Pho85p-Pho80p activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of phosphorylated product/min.

Immunoprecipitation, SDS-PAGE, and Western Blot Analysis—Cell extracts (1 mg of protein) were incubated overnight with 40 μg of anti-Pah1p antibodies (24) and 100 μl of protein A-Sepharose CL-4B beads (10% slurry, w/v) in a total volume of 0.5 ml. Immune complexes were collected by centrifugation at 1,500 × g for 20 s. SDS-PAGE (37) with 8% polyacrylamide gels and Western blotting (38) with polyvinylidene difluoride membrane were performed by standard protocols. Rabbit anti-Pah1p antibodies (24), mouse anti-(phosphoserine/phosphothreonine)-proline (MPM2) antibodies, rabbit anti-phosphatidyserine synthase antibodies (39), and mouse anti-phosphoglycerate kinase antibodies were used at a concentration of 2 μg/ml. Alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies and alkaline phosphatase-conjugated goat anti-mouse IgG antibodies were used at a dilution of 1:5,000. Immune complexes were detected using the enhanced chemifluorescence Western blotting detection kit. Fluorimaging was used to acquire images from Western blots, and the relative densities of the images were analyzed using ImageQuant software. Signals were in the linear range of detectability.

Phosphoamino Acid and Phosphopeptide Mapping Analyses—Recombinant Pah1p was phosphorylated with Pho85p-Pho80p and [γ-32P]ATP, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane. For phosphoamino acid analysis, the 32P-labeled Pah1p on the membrane was subjected to acid hydrolysis with 6 N HCl followed by two-dimensional electrophoresis on cellulose thin-layer chromatography plates (40, 41). For phosphopeptide mapping analysis, 32P-labeled Pah1p on the membrane was subjected to proteolytic digestion with 1:1-tryosylglycine-2-phenethyl chloromethyl ketone-treated trypsin followed by electrophoresis and TLC using cellulose thin-layer chromatography plates (42). Radioactive phosphoamino acids and phosphopeptides were visualized by phosphorimaging analysis, whereas standard phosphoamino acids were visualized by ninhydrin stain.

Mass Spectrometry Analysis of Pah1p Phosphorylation Sites—Recombinant Pah1p was phosphorylated with Pho85p-Pho80p products.
and separated by SDS-PAGE. After in-gel trypsin digestion, peptides were analyzed by matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry to identify phosphopeptide candidates. Based on the phosphopeptide ion inclusion list, quadrupole time-of-flight and Orbitrap liquid chromatography-mass spectrometry/mass spectrometry were performed to identify phosphorylation sites. The mass spectrometry analyses were performed at the Center for Advanced Proteomics Research (University of Medicine and Dentistry of New Jersey, Newark, NJ).

Preparation of [32P]-Labeled PA and Measurement of PAP Activity—The [32P]-labeled PA used for the assay of PAP activity was enzymatically synthesized from DAG and [γ-32P]ATP using *E. coli* DAG kinase (35). PAP activity was measured by following the release of water-soluble [32P]P from chloroform-soluble [32P]PA (10,000 cpm/nmol) (35). The standard reaction contained 50 mM Tris-HCl, 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. 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 PAP activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product/min.

Labeling and Analysis of TAG—Steady-state labeling of TAG with [2,14C]acetate was performed as described previously (43), and lipids were extracted from labeled cells by the method of Bligh and Dyer (44). Lipids were analyzed by one-dimensional thin-layer chromatography on silica gel plates using the solvent system hexane/ethyl ether/acetic acid (40:10:1) (45). The identity of radiolabeled TAG on TLC plates was confirmed by comparison of its migration with that of standard TAG after exposure to iodine vapor. Radiolabeled lipids were visualized by phosphorimaging analysis and quantified using ImageQuant software.

Preparation of Liposomes and Fluorescence Measurements—Liposomes (unilamellar phospholipid vesicles) were prepared with dioleoyl PC and dioleoyl PA at a molar ratio of 10:1 by lipid extrusion (46). Fluorescence measurements were carried out in a Fluoromax-3 fluorometer (HORIBA Jobin Yvon Inc.) at room temperature in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 μg of Pah1p, and the indicated concentrations of liposomes in a total volume of 0.2 ml. The excitation wavelength was 280 nm, and the emission spectra were collected from 300 to 450 nm after a 10-min incubation period. The slit width was set at 5 nm for both excitation and emission beams. The spectra were corrected for light scattering effects by subtracting blanks that contained liposomes but lacked Pah1p.

Analyses of Data—Kinetic data were analyzed according to the Michaelis-Menten and Hill equations using the Enzyme Kinetics module of SigmaPlot software. Dissociation constants for the interaction of Pah1p with PC-PA liposomes were determined as described previously (47). Statistical analyses were performed with SigmaPlot software. The *p* values <0.05 were taken as a significant difference.

**RESULTS**

**Pah1p Is a Bona Fide Substrate of Pho85p-Pho80p with Multiple Phosphorylations on Serine and Threonine Residues**—Proteome-wide phosphorylation studies identified Pah1p as a target of Pho85p-Pho80p (48, 49), which led us to examine the hypothesis that this protein kinase-cyclin complex is responsible for the phosphorylation of the Pah1p sites not phosphorylated by Cdc28p-cyclin B (24). In *vitro* phosphorylation experiments were performed with Pah1p and Pho85p-Pho80p expressed and purified from *E. coli*. The recombinant Pah1p provided a pristine substrate for Pho85p-Pho80p that was free from the endogenous phosphorylations that occur when Pah1p is expressed in yeast (23). Pah1p was incubated with Pho85p-Pho80p in the presence of [γ-32P]ATP, and its phosphorylation was monitored by following the incorporation of the radioactive γ-phosphate into the enzyme. Phosphorimaging analysis of reaction products resolved by SDS-PAGE showed that Pah1p was a substrate for the kinase complex (Fig. 2B). In the absence of the cyclin Pho80p, Pho85p ineffectively phosphorylated...
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Pah1p. Phosphoamino acid analysis of the $^{32}$P-labeled Pah1p showed that Pho85p-Pho80p phosphorylated the protein at both serine and threonine residues with most of the phosphorylation occurring on serine (Fig. 2C). The Pho85p-Pho80p activity was dependent on the time of the reaction, the amount of the kinase complex, and the concentrations of ATP and Pah1p (Fig. 3). The stoichiometry of the phosphorylation reaction was determined from the experiment shown in Fig. 3A where the amount of phosphate incorporated was determined from ImageQuant analysis of the data and a standard curve of $[^\gamma-32P]$ATP. At the point of maximum phosphorylation, Pho85p-Pho80p catalyzed the incorporation of 4 mol of phosphate/mol of Pah1p. This result, coupled to the phosphoamino acid analysis and phosphopeptide mapping analysis (see below), indicated multiple sites of phosphorylation. The dependences of Pho85p-Pho80p activity on ATP and Pah1p followed saturation kinetics and positive cooperative kinetics, respectively (Fig. 3, C and D). Analyses of the data according to the Michaelis-Menten and the Hill equations yielded $K_m$ values for ATP and Pah1p of 3.7 and 0.25 $\mu$M, respectively, and a Hill number for Pah1p of 1.8.

Phosphorylation of Pah1p with Pho85p-Pho80p Attenuates PAP Activity and Interaction with PC-PA Liposomes—If the phosphorylation of Pah1p by Pho85p-Pho80p was physiologically relevant, it might be expected that phosphorylation would affect PAP activity. To address this question, the phosphorylated and unphosphorylated forms of Pah1p were assayed for the dependence of PAP activity on the surface concentration of PA using a Triton X-100/PA mixed micellar substrate. The surface concentration of PA (as opposed to its molar concentration) was varied because PAP activity follows surface dilution kinetics, and under the conditions of these experiments, the activity was independent of the molar concentration of PA (5, 50, 51). As described previously (5), the unphosphorylated enzyme exhibited positive cooperative kinetics with respect to PA (Fig. 4A). The phosphorylation of Pah1p resulted in a decrease in PAP activity (Fig. 4A). Analysis of the data according to the Hill equation indicated that phosphorylation caused a 3-fold decrease in $V_{max}$ and 2-fold increase in $K_m$, and thus the specificity constant for the phosphorylated enzyme was 6-fold lower when compared with the unphosphorylated enzyme (Fig. 4C). The phosphorylation did not have a major effect on the cooperative behavior of the enzyme; the Hill numbers for the unphosphorylated and phosphorylated enzymes were similar (Fig. 4C).

Pah1p is a cytosolic enzyme that interacts with the membrane where its substrate PA resides (5, 24, 25). Studies with
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Phosphorylation-deficient forms of Pah1p have shown that phosphorylation favors a cytosolic location, whereas dephosphorylation allows for a membrane association (24, 25). Pah1p exhibits an inherent fluorescence that increases upon interaction with PC-PA liposomes (52). Accordingly, we used fluorescence spectroscopy to assess the effects of phosphorylation by Pho85p-Pho80p on the interaction of Pah1p with model membranes. As described previously (52), there was a dose-dependent increase in fluorescence of unphosphorylated Pah1p by the addition of PC-PA liposomes (Fig. 4B). Phosphorylation by Pho85p-Pho80p caused a significant decrease in the fluorescence of Pah1p and a 3-fold increase in the dissociation constant (K<sub>d</sub>) for PC-PA liposomes when compared with the control unphosphorylated enzyme (Fig. 4B). These data indicated that Pho85p-Pho80p caused a decrease in the interaction of Pah1p with model membranes.

Ser-110, Ser-114, Ser-602, Thr-723, Ser-744, and Ser-748 Are Phosphorylation Sites of Pho85p-Pho80p—O’Hara et al. (23) have identified seven phosphorylation sites in Pah1p that are targets for proline-directed Ser/Thr protein kinases (Fig. 5A). Three of these sites (Ser-602, Thr-723, Ser-744) are targets of Cdc28p-cyclin B (24). We examined the hypothesis that the four sites not phosphorylated by Cdc28p-cyclin B are targets of proline-directed Ser/Thr protein kinases (23). The values reported are the average of three experiments ± S.D. (error bars). B, the unphosphorylated and phosphorylated forms of Pah1p were incubated with the indicated concentrations of PC-PA liposomes. Following a 10-min incubation, the increase in Pah1p fluorescence was measured. The data are plotted with respect to total phospholipid concentration in the liposomes. The values reported are the average of three experiments ± S.E. (error bars). C. The V<sub>max</sub>, K<sub>m</sub>, and Hill values were determined from the data in A, and the K<sub>v</sub> values were determined from the data in B.

FIGURE 4. Phosphorylation of Pah1p with Pho85p-Pho80p attenuates PAP activity and interaction with PC-PA liposomes. Purified recombinant Pah1p (1 μg) was phosphorylated with Pho85p-Pho80p complex (1 μg). A, PAP activity of the unphosphorylated and phosphorylated forms of the enzyme was measured as a function of the surface concentration (mol %) of PA. The molar concentration of PA was held constant at 0.2 mM, and the Triton X-100 concentration was varied to obtain the indicated surface concentrations. The values reported are the average of three experiments ± S.D. (error bars). B, the unphosphorylated and phosphorylated forms of Pah1p were incubated with the indicated concentrations of PC-PA liposomes. Following a 10-min incubation, the increase in Pah1p fluorescence was measured. The data are plotted with respect to total phospholipid concentration in the liposomes. The values reported are the average of three experiments ± S.E. (error bars). C. The V<sub>max</sub>, K<sub>m</sub>, and Hill values were determined from the data in A, and the K<sub>v</sub> values were determined from the data in B.

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Phosphorylation of wild type Pah1p caused a 73% reduction in PAP activity, and this inhibitory effect was diminished (13–33%) by each of the Ser/Thr to alanine mutations (Fig. 6). Thus, phosphorylation of each of the sites contributed to the inhibitory effects of Pho85p-Pho80p on PAP activity. The 7A mutation (all seven sites mutated to alanine) essentially eliminated the inhibitory effect that Pho85p-Pho80p had on PAP activity (Fig. 6). The 7A mutation also eliminated the inhibitory effect that Pho85p-Pho80p had on the interaction of Pah1p with PC-PA liposomes (data not shown). The K<sub>v</sub> values for the Pah1p-7A mutant enzyme without and with Pho85p-Pho80p treatment were 0.13 and 0.14 mM, respectively.

pho85<sup>Δ</sup> Mutation Affects Phosphorylation, Abundance, and Location of Pah1p in Vivo—We sought evidence that Pah1p was phosphorylated by Pho85p-Pho80p in vivo. To address this issue, Pah1p was immunoprecipitated from wild type and pho85<sup>Δ</sup> mutant cells with anti-Pah1p antibodies followed by Western blot analysis of the immune complexes with anti-phosphoserine/phosphothreonine)-proline (MPM2) antibodies (Fig. 7A). The presence of Pah1p in the immune complex was confirmed by Western blotting with anti-Pah1p antibodies, and the specificity of the MPM2 antibodies for phosphorylated Pah1p was confirmed with purified Pah1p that was phosphorylated with purified Pho85p-Pho80p (Fig. 7A). The experiment with purified Pah1p also showed that phosphorylation caused a reduction in the electrophoretic mobility of the enzyme, which has been attributed to phosphorylation of Thr-723 (23, 24). In wild type cells, the immunoprecipitated Pah1p reacted with the MPM2 antibodies, indicating that the enzyme was phosphorylated in vivo by a kinase(s) with specificity for a (Ser/Thr)-Pro motif. The MPM2 antibodies could barely detect phosphorylated Pah1p in the immune complex derived from pho85<sup>Δ</sup> mutant cells, suggesting that Pah1p was phosphorylated in vivo by Pho85p-Pho80p. However, the interpretation of this experiment was complicated by the fact that the amount of Pah1p in the mutant was much reduced when compared with the wild type control (Fig. 7A). The effect of Pho85p-Pho80p phosphorylation and Pah1p abundance was examined further. It is known that Pho85p-Pho80p is enzymatically active when phosphate is replete in the growth medium, but the kinase complex is inactivated by the inhibitor Pho81p when phosphate is depleted from the growth medium (53). The abundance of Pah1p was low in wild type cells grown in low phosphate, whereas the abundance increased when phosphate supplementation (Fig. 7B). Western blot analysis with anti-Pah1p
antibodies showed that the amount of Pah1p in pho85/H9004 mutant cells was reduced by 60% when compared with wild type cells (Fig. 7C). The cell extracts derived from wild type and pho85/H9004 mutant cells were fractionated into cytosolic and membrane fractions followed by Western blot analysis with anti-Pah1p antibodies. In wild type cells, 16% of Pah1p was associated with membranes, whereas in pho85/H9004 mutant cells, 78% of Pah1p was associated with membranes (Fig. 7D). Thus, although the total amount of Pah1p was reduced in the pho85/H9004 mutant, a much greater amount of the enzyme was associated with membranes when compared with the cytosol.

Pho85p-Pho80p Phosphorylation Site Mutations Affect Pah1p Abundance, TAG Content, and Location of Pah1p in Vivo—The physiological consequences of the Pho85p-Pho80p phosphorylation site mutations in Pah1p were examined. For these experiments, wild type and Ser/Thr to alanine mutant proteins were expressed from a low copy number plasmid in pah1/H9004 nem1/H9004 mutant cells. As discussed previously (24), the reason for using the nem1/H9004 mutant background was to assess the dependence of Pah1p function on the Nem1p-Spo7p protein phosphatase complex and to examine the phosphorylation site mutations in a genetic background that favored the phosphorylation of other non-mutated phosphorylation sites in Pah1p. Indeed, Nem1p-Spo7p-dependent dephosphorylation is required for Pah1p function in vivo, but this requirement can be circumvented by a phosphorylation-deficient form of the enzyme (17, 24, 25).

The expression levels of the phosphorylation site mutant forms of Pah1p were determined by Western blot analysis of cell extracts, and the ImageQuant analyses of the data are presented in Fig. 8A. Many of the mutations affected the abundance of Pah1p. Individually, the S110A, S114A, and S168A mutations had the greatest effects on abundance, causing a reduction in the amounts of Pah1p by about 30%. The 3A mutation (combination of the sites phosphorylated by both Pho85p-Pho80p and Cdc28p-cyclin B) caused a 25% reduction in Pah1p abundance, whereas the 4A mutation (combination of sites spe-
Specific to Pho85p-Pho80p phosphorylation) caused a 40% reduction in the abundance of the protein (Fig. 8A). As described previously (24), the 7A mutation caused a 50% reduction in the abundance of Pah1p. To further examine the relationship between Pho85p-Pho80p phosphorylation and Pah1p abundance, a Western blot analysis was performed on pah1Δ and pah1Δ nem1Δ cells expressing the wild type and 7A mutant forms of the enzyme. The effects of phosphorylation on Pah1p abundance were readily observed when samples were analyzed from cultures in the early to late exponential phase of growth (Fig. 8B). When wild type Pah1p was expressed in pah1Δ mutant cells (with Nem1p-Spo7p), the levels of protein decreased as cells grew from 12 to 18 h. However, when expressed in pah1Δ nem1Δ mutant cells (lacking the protein phosphatase complex), the levels of wild type Pah1p were more abundant (Fig. 8B). As discussed above, the abundance of the 7A mutant enzyme, which can bypass the requirement for Nem1p-Spo7p dephosphorylation (17, 24, 25), was less abundant in pah1Δ and pah1Δ nem1Δ cells when compared with wild type Pah1p (Fig. 8B).

The PAP activity of Pah1p plays a major role in the synthesis of TAG (5, 6), and thus, we examined the effects of the phosphorylation site mutations on this function in lipid metabolism. Cells were labeled to steady state with [2-13C]acetate followed by the extraction and analysis of TAG. As described previously (24), the TAG content of pah1Δ nem1Δ mutant cells expressing wild type Pah1p was about 4% (Fig. 8C). This relatively low amount of TAG3 indicated the importance of Nem1p-Spo7p

3 The TAG content of pah1Δ cells expressing wild type Pah1p is 22% (24).
Phosphorylation for Pah1p function in lipid metabolism (24). The phosphorylation site mutations (individually and in combination) caused increases in TAG content (Fig. 8C), substantiating that phosphorylation-deficient forms of Pah1p can bypass the requirement of Nem1p-Spo7p dephosphorylation (24). Of the individual mutations, S110A and S748A had the greatest effects, causing an increase in TAG content of 2.3- and 3-fold, respectively. The effect of the 4A mutation on TAG content (5-fold increase) was similar to that of the 7A mutation, whereas the effect of the 3A mutation was similar to that observed for the S110A and S748A mutations alone (Fig. 8C).

The relative abundance of Pah1p in the cytosol and membrane fractions of pah1Δ nem1Δ mutant cells expressing the 3A, 4A, and 7A mutations was examined (Fig. 8D). As described previously (24), the amounts of membrane-associated Pah1p in cells expressing the 3A and 7A mutants were 2- and 5-fold greater, respectively, when compared with cells expressing the wild type Pah1p. The 4A mutation caused a 3.7-fold increase in the membrane association of Pah1p. Thus, although the phosphorylation site mutations caused a decrease in Pah1p abundance, a greater amount of the dephosphorylated form of the enzyme was associated with membranes, and this correlated with the increased synthesis of TAG.

DISCUSSION

Phosphorylation/dephosphorylation and cellular location are major mechanisms by which the physiological functions (i.e. PAP activity, control of cellular levels of PA and DAG, and TAG synthesis) of Pah1p are regulated (2, 3, 23–25). Activation of Pah1p is mediated by Nem1p-Spo7p dephosphorylation that occurs at the nuclear/ER membrane, whereas the inhibition of function is mediated by phosphorylation that presumably occurs in the cytosol (17, 23–25) (Fig. 1). In this work, we advanced understanding of Pah1p phosphorylation and identified Pho85p-Pho80p as a relevant protein kinase complex that regulated PAP activity, its cellular location and abundance, and function in the synthesis of TAG.

Pah1p has been known to be subject to multiple phosphorylations (23), and in particular, the seven sites contained within the (Ser/Thr)-Pro motif play a major role in Pah1p regulation (23–25). Support for this assertion has come from the analysis of the Pah1p-7A mutant enzyme where all seven sites are simultaneously mutated to alanine residues. The 7A mutation causes a 1.8-fold increase in PAP activity (23) and circumvents the Nem1p-Spo7p requirement for dephosphorylation that is needed for Pah1p function and TAG synthesis at the membrane (23, 24). In addition, the expression of the activated Pah1p-7A mutant enzyme reduces growth on medium lacking inositol, a result caused by activation of Opi1p repressor activity (which is normally inhibited by its interaction with PA at the nuclear/ER membrane (54)) on the inositol biosynthesis gene INO1 (23).

Proteome-wide phosphorylation studies have shown that Pah1p is phosphorylated by the cyclin-dependent protein kinases Cdc28p (55) and Pho85p (48, 49). These proline-directed Ser/Thr protein kinases are known to play roles in cell cycle progression, gene expression, macromolecular metabolism, and signaling in response to environmental conditions (26, 27, 56). Our previous studies have shown that among the seven (Ser/Thr)-Pro sites Cdc28p-cyclin B phosphorylated Pah1p on Ser-602, Thr-723, and Ser-744 (24). However, these phosphorylations have little effect on Pah1p activity and TAG synthesis (24). Thus, phosphorylations of the remaining sites had been predicted to be responsible for the major effects imparted by the 7A mutation (24).

Pho85p was identified as the protein kinase responsible for the phosphorylations of the remaining sites. In fact, the Pho85p-Pho80p complex phosphorylated the three Cdc28p sites as well, an event that is not unusual for substrates common to both cyclin-dependent protein kinases (28). Indeed, Pho85p and Cdc28p share 52% sequence identity. The phosphorylation efficiency (as reflected in stoichiometry) was much greater for Pho85p-Pho80p (4 mol of phosphate/mol of Pah1p) when compared with Cdc28p-cyclin B (0.8 mol of phosphate/mol of Pah1p). In the absence of Pho80p, the phosphorylation of Pah1p by Pho85p was negligible. Pho85p associates with and is activated by nine additional cyclins (known as Pcls for Pho85p cycling) (27, 28). Although we did not test other Pcls for their ability to support the phosphorylation of Pah1p by Pho85p, a global analysis of protein phosphorylation indicates that phosphorylation of Pah1p by Pho85p-Pho80p is 6–7-fold greater when compared with the phosphorylation by Pho85p-Pcl1p and Pho85p-Pcl9p (48), complexes known to affect cell cycle progression and morphogenesis (27). Nonetheless, our studies clearly demonstrated that Pah1p was a bona fide substrate of Pho85p-Pho80p with Kₐ values for Pah1p and ATP in the low micromolar range. Importantly, the Pho85p-Pho80p phosphorylation of Pah1p affected the biochemical properties of the enzyme; phosphorylation reduced by 6-fold the catalytic efficiency (Vₘₐₓ/Kₐ) of PAP activity and reduced by 3-fold the association (Kₐ) with PC-PA liposomes. Moreover, the 7A mutation ablated the inhibitory effect Pho85p-Pho80p had on PAP activity with each of the seven mutations having a partial effect. Likewise, the 7A mutation prevented the inhibitory effect Pho85p-Pho80p had on Pah1p interaction with PC-PA liposome membranes.

The analysis of wild type Pah1p in pho85Δ and in nem1Δ pah1Δ mutant cells coupled to the analysis of the phosphorylation-deficient forms of Pah1p indicated that Pho85p-Pho80p phosphorylation affected Pah1p abundance. Pah1p was more abundant in cells lacking the Nem1p-Spo7p complex, and as the number of phosphorylation site mutations increased, the less abundant Pah1p became. When Pho85p-Pho80p activity was inactivated in wild type cells by phosphate depletion, the abundance of Pah1p was greatly reduced, but when phosphate was replenished, Pah1p became abundant. This experiment, however, does not rule out the possibility that cellular phosphate might also regulate Pah1p expression by a genetic mechanism. With respect to phosphorylation and Pah1p abundance, there are numerous examples where phosphorylation stabilizes protein abundance (57–59). Like Pah1p, phosphorylation of the phosphoinositide phosphatase (encoded by PTEN) stabilizes protein abundance, whereas dephosphorylation leads to loss of protein abundance but at the same time causes an increase in phosphoinositide phosphatase activity (57). In the case of Pah1p, the loss of enzyme abundance after dephosphorylation and activation may be a mechanism to attenuate enzyme func-
tion at the membrane. The need for this type of regulation may be supported by the fact that overexpression of the activated 7A mutant enzyme causes a loss of growth phenotype (24). The mechanism that controls the abundance of dephosphorylated Pah1p is unknown and will be the subject of future studies.

The cell fractionation studies of the wild type and phosphorylation-deficient forms of Pah1p supported the conclusion reached by the in vitro studies that Pho85p-Pho80p phosphorylation governed the association of Pah1p with membranes. Although loss of phosphorylation caused a decrease in Pah1p abundance, a greater amount of the enzyme was associated with membranes when compared with phosphorylated enzyme. Moreover, the Pho85p-Pho80p phosphorylation site mutations allowed bypass of the Nem1p-Spo7p requirement for dephosphorylation and Pah1p function at the membrane for TAG synthesis.

Pho85p-Pho80p phosphorylation is known for its involvement in the regulation of phosphate metabolism (28, 60). In brief, low phosphate leads to the Pho81p-mediated inhibition of Pho85p-Pho80p activity and the induction of PHO genes and their products for foraging phosphate from the environment (60). When phosphate levels are replete, Pho85p-Pho80p is active to inhibit expression of PHO genes (28, 60). Whether or not inactivation of Pho85p-Pho80p phosphorylation of Pah1p and activation of its PAP activity to generate free phosphate from PA are a means to control cellular phosphate is unknown. However, it is known that PA levels are not significantly affected by phosphate starvation (61). On the other hand, the levels of lyso-PA (the immediate precursor of PA having one less fatty acyl tail) are drastically reduced under phosphate-starved conditions, and the dephosphorylation of lyso-PA is attributed to the induced expression of Phm8p lyso-PA phos-

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