Phosphatidate Phosphatase Plays Role in Zinc-mediated Regulation of Phospholipid Synthesis in Yeast*

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Background: In yeast, phospholipid synthesis is regulated by zinc deficiency.

Results: PAH1-encoded phosphatidate phosphatase activity was induced in zinc-deficient cells by a transcriptional mechanism.

Conclusion: The zinc-mediated regulation of phosphatidate phosphatase affects phospholipid synthesis by controlling phosphatidate and diacylglycerol.

Significance: The transcriptional regulation of phosphatidate phosphatase plays an important role in controlling phospholipid synthesis by zinc.

In the yeast Saccharomyces cerevisiae, the synthesis of phospholipids is coordinately regulated by mechanisms that control the homeostasis of the essential mineral zinc (Carman, G.M., and Han, G. S. (2007) Regulation of phospholipid synthesis in Saccharomyces cerevisiae by zinc depletion. Biochim. Biophys. Acta 1771, 322–330; Eide, D. J. (2009) Homeostatic and adaptive responses to zinc deficiency in Saccharomyces cerevisiae. J. Biol. Chem. 284, 18565–18569). The synthesis of phosphatidylcholine is balanced by the repression of CDP-diacylglycerol pathway enzymes and the induction of Kennedy pathway enzymes. PAH1-encoded phosphatidate phosphatase catalyzes the penultimate step in triacylglycerol synthesis, and the diacylglycerol generated in the reaction may also be used for phosphatidylcholine synthesis via the Kennedy pathway. In this work, we showed that the expression of PAH1-encoded phosphatidate phosphatase was induced by zinc deficiency through a mechanism that involved interaction of the Zap1p zinc-responsive transcription factor with putative upstream activating sequence zinc-responsive elements in the PAH1 promoter. The pah1Δ mutation resulted in the derepression of the CHO1-encoded phosphatidylserine synthase (CDP-diacylglycerol pathway enzyme) and loss of the zinc-mediated regulation of the enzyme. Loss of phosphatidate phosphatase also resulted in the derepression of the CKI1-encoded choline kinase (Kennedy pathway enzyme) but decreased the synthesis of phosphatidylcholine when cells were deficient of zinc. This result confirmed the role phosphatidate phosphatase plays in phosphatidylcholine synthesis via the Kennedy pathway.

In the yeast Saccharomyces cerevisiae, the essential mineral zinc serves as a catalytic or structural cofactor for many enzymes and transcription factors (1). Accordingly, cells have developed mechanisms to maintain internal stores of zinc (e.g. induced expression of zinc transporters) when extracellular zinc is limiting (1). In addition, cells require mechanisms to adapt physiological processes to limiting amounts of zinc, and one of these processes is the synthesis of membrane phospholipids (1, 2).

The major membrane phospholipids in yeast include PC, PE, PI, and PS (3, 4). PC and PE are synthesized (de novo) from the phospholipid precursor PA by complementary CDP-DAG and Kennedy pathways (see Fig. 1) (4). In the CDP-DAG pathway, PC and PE are derived from PA via CDP-DAG, whereas in the Kennedy pathway, these phospholipids are derived from PA via DAG (Fig. 1). PI and PS are only derived from PA via CDP-DAG (Fig. 1). Zinc limitation causes changes in cellular phospholipid composition that include a decrease in PE content and an increase in PI content (5). The decrease in PE is attributed to reductions in both PS synthase and PE decarboxylase activities (which produce PS and PE, respectively, in the CDP-DAG pathway), whereas the increase in PI is attributed to an elevation in PI synthase activity (5, 6). Despite the fact that CDP-DAG pathway activities (including PE/phospholipid methyltransferases that convert PE to PC) are repressed in response to zinc depletion, the PC content is not significantly affected (5). Maintaining PC content in response to zinc depletion is attributed to the induced expression of choline kinase (7) and ethanolamine kinase (8),3 the enzyme activities that catalyze the committed steps in the Kennedy pathway (Fig. 1).

The zinc-mediated regulation of some phospholipid synthesis enzymes has been ascribed to transcriptional mechanisms (1, 2, 4) (Fig. 1). The repression of CHO1 (for PS synthase) is mediated by the PA-regulated transcriptional repressor Opi1p (5, 9), whereas the inductions of PIS1 (for PI synthase), CKII (for choline kinase), and EKI1 (for ethanolamine kinase) are mediated by the zinc-sensing and zinc-inducible transcriptional activator Zap1p (6–8, 10, 11). According to the model shown in Fig. 1, Zap1p, which is induced by zinc depletion (10,

2 The abbreviations used are: PC, phosphatidylcholine; PA, phosphatidate; DAG, diacylglycerol; TAG, triacylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; DAG, diacylglycerol; ZRE, zinc-responsive element; UASZRE, upstream activating sequence zinc-responsive element; ER, endoplasmic reticulum.

3 Although the induction of ethanolamine kinase results in elevated amounts of ethanolamine-containing Kennedy pathway intermediates, the steady-state PE content does not change because PE is methylated to form PC (8).
Supplementation, a growth condition that represses the expression of cho genes (9, 13–15). This regulation occurs in the absence of inositol (5). The activity of PA phosphatase was induced by zinc deficiency through a mechanism that involved Zap1p (7), and the expression of PA phosphatase was regulated in response to zinc status. In this work, we found that the expression of PAH1-encoded PA phosphatase catalyzes the dephosphorylation of PA to produce DAG and P_i (18). A reduction in the content of PA (which tethers Opi1p and stabilizes its association with Scs2p at the nuclear/ER membrane (9, 12)), which is caused by the increased synthesis of PI via CDP-DAG, leads to the translocation of Opi1p into the nucleus where it interacts with Ino2p to attenuate the transcriptional activation of CHO1 by the Ino2p-Ino4p complex (9, 13–15). This regulation occurs in the absence of inositol supplementation, a growth condition that represses the expression of cho genes (4, 15–17).

The yeast PAH1-encoded PA phosphatase catalyzes the dephosphorylation of PA to produce DAG and P_i (18). The DAG generated by this reaction is used for the synthesis of TAG and may also be used for the synthesis of PE and PC via the Kennedy pathway (19, 20) (Fig. 1). The activity of PA phosphatase also controls PA content and the transcriptional regulation of UAS_{INO}-containing phospholipid synthesis genes (21, 22). Given these roles in phospholipid synthesis, we hypothesized that PA phosphatase was regulated in response to zinc status. In this work, we found that the expression of PAH1-encoded PA phosphatase was induced by zinc deficiency through a mechanism that involved Zap1p. Moreover, loss of Pah1p PA phosphatase resulted in the derepression of both choline kinase and PS synthase activities and loss of the zinc-mediated regulation of PS synthase. A PA phosphatase activity, whose gene(s) has yet to be identified, was also induced in response to zinc depletion through a mechanism that involved Zap1p.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were reagent grade. Growth medium supplies were from Difco, and yeast nitrogen base lacking zinc sulfate was purchased from BIO 101. Restriction endonucleases, modifying enzymes, and the NEBlot kit were purchased from New England Biolabs. The Yeastmaker™ yeast transformation kit was obtained from Clontech. Plasmid DNA purification and DNA gel extraction kits were from QiaGen. Oligonucleotides for PCRs and electrophoretic mobility shift assays were prepared by Genosys Biotechnology, Inc. ProbeQuant G-50 columns were purchased from GE Healthcare. DNA markers for agarose gel electrophoresis and protein assay reagents were purchased from Bio-Rad. Ampicillin, chloramphenicol, benzamidine, bovine serum albumin, choline, phosphocholine, CDP-choline, leupeptin, O-nitrophenyl β-D-galactopyranoside, pepstatin, phenylmethylsulfonyl fluoride, IGE-PAL CA-630, and Triton X-100 were purchased from Sigma. Lipids were purchased from Avanti Polar Lipids. Silica gel 60 thin layer chromatography plates were from EM Science. Radiochemicals and scintillation counting supplies were purchased from PerkinElmer Life Sciences and National Diagnostics, respectively. Liqul-Now detergent was from Alconox, Inc.

**Strains, Plasmids, and Growth Conditions**—The strains and plasmids used in this work are listed in Table 1. Plasmid pFP1 contains the PAH1 promoter fused to the coding sequence of the lacZ gene of Escherichia coli. This plasmid was constructed by replacing the DPP1 promoter in pJO2 (23) with the PAH1 promoter sequence at the KpnI/EcoRI site. The PAH1 pro-
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TABLE 1

<table>
<thead>
<tr>
<th>Strains or plasmid</th>
<th>Relevant characteristics</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>Strains E. coli</td>
<td>DH5α (F · φ80lacZAM15 Δ[lacZYA-argF]U169 deoR recA1 endA1 hisD17(r6K− mcrA−) phoA supE44 thi−1 gyrA96 relA1)</td>
<td>25</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>W303-1A (MATa ade2−1 can1−100 his3−11,15 leu2−3,112 trpl−1 ura3−1)</td>
<td>71</td>
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<tr>
<td>GHY57 pah1Δ·URA3 derivative of W303-1A</td>
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<tr>
<td>TBY1 dpp1Δ·TRP1[Kan−] ppp1Δ·HIS3[Kan−] derivative of W303-1A</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>DY1457 (MATa ade6 can1−100 his3−11,15 leu2−3,112 trpl−1 ura3−52)</td>
<td>72</td>
<td></td>
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<tr>
<td>ZHY3 zrt1Δ·LEU2 zrt2Δ·HIS3 derivative of DY1457</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>ZHY16 (MATa ade6 can1−100 his3·leu2 ura3 zap1Δ·TRP1)</td>
<td>72</td>
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<tr>
<td>Plasmids pJO2 Pcpp-rac reporter gene containing the DPP1 promoter with URA3</td>
<td>23</td>
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<tr>
<td>pFP1 Ppp-rac reporter gene containing the PAH1 promoter with URA3</td>
<td>This study</td>
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<tr>
<td>pPAH1-ZRE2 Derivative of pFP1 with mutations in ZRE2</td>
<td>This study</td>
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motor was obtained by PCR (24) with the primers 5′-GCGGGATCCATACTCACGACCC-3′ (forward) and 5′-GCGGGATCCATACTCACGACCC-3′ (reverse) using strain W303-1A genomic DNA as the template. The PCR primer used in the forward direction corresponds to −1000 bp from the start codon, and the primer used in the reverse direction corresponds to +3 bp from the start codon. The correct orientation of the promoter was confirmed by restriction enzyme digestion. Plasmid pPAH1-ZRE2 was a derivative of pFP1 in which the putative UASZRE sequence ZRE2 in the PAH1 promoter was mutated to a nonconsensus sequence, 5′-AAAAAAAAAAAAAAA-3′. Plasmid and genomic DNA preparation, restriction enzyme digestion, and DNA ligations were performed by standard methods (25). Plasmid maintenance and amplification were performed in E. coli strain DH5α. Transformation of plasmids into E. coli (25) or S. cerevisiae (26) was performed as described previously.

*S. cerevisiae* cells were grown in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or in synthetic complete medium (27) containing 2% glucose at 30 °C. The appropriate amino acids of synthetic complete medium were omitted for selection purposes. Zinc-deficient medium was synthetic complete medium (27) prepared with yeast nitrogen base lacking zinc sulfate. Standard synthetic complete medium contains 1.5 μM zinc sulfate. The internal stores of zinc were depleted from cells by growth in zinc-deficient medium (28). To confirm that the intracellular levels of zinc were depleted, we made use of the P<sub>CYCI-ZRE</sub>-lacZ reporter gene assay described by MacDiamid et al. (29). The synthetic complete medium lacked inositol supplementation to preclude the regulatory effects that this phospholipid precursor molecule has on the regulation of phospholipid metabolism (4, 30−32). For growth on plates, the growth media were supplemented with 2% agar. Yeast cell numbers in liquid medium were determined spectrophotometrically at an absorbance of 600 nm. Exponential phase cells were harvested at a density of ~0.5 × 10<sup>7</sup> cells/ml. Glassware was washed with Liqui-Nox, rinsed with 0.1 mM EDTA, and then rinsed several times with deionized distilled water to remove zinc contamination. E. coli cells were grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) at 37 °C. Ampicillin (100 μg/ml) was added to bacterial cultures that carried plasmids.

Preparation of Cell Extracts, Cytosolic Fraction, and Protein Determination—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-Bead-Beater-16 (33). The cell disruption buffer contained 50 mM Tris-HCl, pH 7.5, 0.3 M 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 cytosolic (supernatant) fraction was prepared by centrifugation at 100,000 × g for 1 h (33). Protein concentration was estimated by the Coomassie Blue dye binding assay of Bradford (34) using bovine serum albumin as the standard.

Enzyme Assays—All assays were conducted in triplicate at 30 °C. β-Galactosidase activity was measured by following the formation of O-nitrophenyl from O-nitrophenyl β-D-galactopyranoside spectrophotometrically at a wavelength of 410 nm (35). The assay mixture contained 100 mM sodium phosphate, pH 7.0, 3 mM O-nitrophenyl β-D-galactopyranoside, 1 mM MgCl<sub>2</sub>, 100 mM 2-mercaptoethanol, and enzyme protein in a total volume of 0.1 ml. PA phosphatase activity was measured by following the release of water-soluble <sup>32</sup>P<sub>2</sub> from chloroformsoluble [32P]PA (10,000 cpm/nmol) (33). The reaction mixture contained 50 mM Tris-HCl buffer, pH 7.5, 5 mM MgCl<sub>2</sub>, 0.2 mM PA, 2 mM Triton X-100, and enzyme protein in a total volume of 0.1 ml. Choline kinase activity was measured by following the incorporation of [methyl-<sup>14</sup>C]choline (2,000 cpm/nmol) into radiolabeled phosphocholine after precipitation of the labeled choline as a reineckate salt (36). The assay mixture contained 67 mM glycine-NaOH buffer, pH 9.5, 5 mM choline, 5 mM ATP, 10 mM MgSO<sub>4</sub>, 1.3 mM dithiothreitol, and enzyme protein in a total volume of 0.06 ml (37). PS synthase activity was measured by following the incorporation of 0.5 mM [3-<sup>3</sup>H]serine (10,000 cpm/nmol) into PS in the presence of 50 mM Tris-HCl, pH 8.0, 0.6 mM MnCl<sub>2</sub>, 3.2 mM Triton X-100, and 0.2 mM CDP-DAG in a total volume of 0.1 ml (38). All assays were linear with time and protein concentration. The average standard deviation of all assays was ±5%. The units of β-galactosidase and choline kinase activities were defined as the amount of enzymes that catalyzed the formation of 1 μmol of product/min. The units of PA phosphatase and PS synthase activities were defined in nmol of product/min. Specific activity was defined as units/mg of protein.

Electrophoretic Mobility Shift Assays—Double-stranded oligonucleotides (Table 2) were prepared, labeled with [α-<sup>32</sup>P]dATP (400−800 Ci/mmole) and Klenow fragment (5 units), and then purified by gel filtration using ProbeQuant G-50 spin columns as described previously (7). Purified recom-
binant GST-Zap1p (687–880) (6) was incubated with 1 pmol of radiolabeled DNA probe (2.0 × 10^5 cpm/pmol) for 15 min at room temperature in a total volume of 10 μl. The reaction buffer contained 10 mM Tris- HCl, pH 8.0, 10 mM MgCl₂, 50 mM KCl, 1 mM dithiothreitol, 0.025 mg/ml poly(dI-dC)·poly(dI·dC), 0.2 mg/ml bovine serum albumin, 0.04% IGEPA LA CA-630, and 10% glycerol. Following incubation, the reaction mixture was resolved on a 15% polyacrylamide gel (1.5-mm thickness) in 0.5× Tris borate-EDTA buffer at 100 V for 45 min. Gels were dried onto blotting paper, and the radioactive signals were visualized by phosphorimaging analysis.

Labeling and Analysis of Lipids and Kennedy Pathway Intermediates—Exponential phase cells were labeled for five to six generations with either 0.2 μCi/ml [methyl-¹⁴C]choline (for Kennedy pathway intermediates and PC) or 1 μCi/ml [2,14C]acetate (for neutral lipids) (39). Lipids and the Kennedy pathway intermediates were extracted from cells using chloroform/methanol/water followed by separation of the chloroform and aqueous phases (40). The chloroform and aqueous phases that contained the lipids and Kennedy pathway intermediates, respectively, were dried in vacuo and then dissolved in 100 μl of chloroform and 100 μl of methanol/water (1:1, v/v), respectively (39). The Kennedy pathway intermediates were subjected to TLC on silica gel plates using the solvent system methanol, 0.6% sodium chloride, ammonium hydroxide (10:10:1, v/v). PC was analyzed by TLC on silica gel plates using the solvent system chloroform, pyridine, 88% formic acid, methanol, water (60:35:10:5.2, v/v). Neutral lipids were analyzed by TLC on silica gel plates using the solvent system hexane/diethyl ether/glacial acetic acid (40:10:1, v/v). The identity of the labeled lipids and Kennedy pathway intermediates on TLC plates was confirmed by comparison with standards. Radiolabeled compounds were visualized by phosphorimaging analysis, and the relative quantities of labeled lipids were analyzed using ImageQuant software.

Analyses of Data—The Student’s t test was used to assess statistical significance and was performed with SigmaPlot software. The p values < 0.05 were taken as a significant difference.

RESULTS

Zinc Deficiency Causes Zap1p-dependent Induction of PA Phosphatase Activity—We addressed the hypothesis that PAH1-encoded PA phosphatase was regulated by zinc deficiency. Activity was first measured from wild type cells. The standard assay mixture contained Mg²⁺ ions that are needed for Pah1p activity (18). The concentration of Mg²⁺ (5 mM) used in the assay does not have a major effect on the DPP1- and LPP1-encoded PA phosphatase activities that do not have a Mg²⁺ requirement (41–44). Removal of zinc from the growth medium resulted in a 4.3-fold increase in PA phosphatase activity (Fig. 2A). This increase in activity could be attributed in part to Dpp1p, which is known to be induced by zinc deficiency (28).

We next examined the effect of zinc deficiency on PA phosphatase activity in dpp1Δ lpp1Δ mutant cells that lack both Dpp1p and Lpp1p (Fig. 2A). In zinc-deficient medium, the activity in the double mutant was 50% lower than that of wild type cells. Furthermore, PA phosphatase activity in the dpp1Δ lpp1Δ mutant grown without zinc was 3-fold higher when compared with the same cells grown in zinc-replete medium. Thus, the attenuated expression of PA phosphatase activity in dpp1Δ lpp1Δ mutant cells deficient of zinc was consistent with the loss of DPP1. Moreover, the induced expression of PA phosphatase activity in zinc-deficient dpp1Δ lpp1Δ mutant cells indicated that the activity encoded by PAH1 was also regulated by zinc status.

The PAH1 promoter contains three putative UAS_{ZRE} sequences (see below) that are potential binding sites for the Zap1p transcription factor (11). Zap1p, which itself is induced by zinc depletion (10), interacts with UAS_{ZRE} sequences in the promoters of several phospholipid synthesis genes (e.g. DPP1, CKI1, EKI1, and PIS1) to activate transcription when cells are deficient of zinc (6–8, 28). To address whether the zinc-mediated regulation of the Mg²⁺-dependent PA phosphatase enzymes was mediated by Zap1p, we measured activity in the zap1Δ mutant. The cytosolic fractions of wild type and zap1Δ mutant cells were utilized for this experiment. In doing so, we
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eliminated the PA phosphatase activities attributed to DPP1 and LPP1 because their products are associated with the membrane fraction (41, 43). Moreover, the PA phosphatase assay was performed in the presence of N-ethylmaleimide to differentiate between the Mg$^{2+}$-dependent activities encoded by PAH1 and an unknown gene(s) (18). When the assay was performed in the absence of N-ethylmaleimide, the activity was a reflection of PA phosphatase encoded by PAH1 and the unknown gene(s), whereas the activity measured in the presence of N-ethylmaleimide was a reflection of the PAH1-encoded enzyme (18). Indeed, the PA phosphatase activity in the cytosolic fraction of wild type cells that were grown in zinc-replete medium was reduced by 25% when N-ethylmaleimide was present in the assay (Fig. 2B). The deficiency of zinc in the growth medium resulted in a 3.2- and 2.6-fold increase in PA phosphatase activity when the assays were performed in the absence and presence of N-ethylmaleimide, respectively (Fig. 2B). Thus, both PA phosphatase activities were regulated in response to zinc status. That this regulation was practically eliminated in the zap1Δ mutant (Fig. 2B) supported the conclusion that the PA phosphatase activities encoded by PAH1 and the unknown gene(s) were induced by zinc deficiency in a Zap1p-dependent manner.

Deficiency of Cytosolic Zinc Causes Zap1p-mediated Induction of P_PAH1-lacZ Reporter Gene Activity—The effect of zinc deficiency on the expression of the PAH1 gene was examined by use of a P_PAH1-lacZ reporter gene in which the PAH1 promoter was fused in-frame with the coding sequence of the E. coli lacZ gene, and β-galactosidase activity was dependent on the transcription of lacZ driven by the PAH1 promoter. The analysis of β-galactosidase activity from extracts derived from wild type cells grown in the absence and presence of zinc indicated that PAH1 expression was induced in response to zinc deficiency (Fig. 3A). The β-galactosidase activity in zinc-deficient cells was 6-fold greater than the activity found in cells grown with 1.5 μM zinc. The expression of PAH1 was also examined in the zrt1Δ zrt2Δ mutant that lacks both the high (Zrt1p) and low (Zrt2p) affinity plasma membrane zinc transporters (45, 46). This mutant contains a low cytosolic level of zinc despite excess zinc supplementation in the growth medium (45, 46). That the expression of P_PAH1-lacZ reporter gene in the zrt1Δ zrt2Δ mutant grown with 1.5 μM zinc was elevated to the same extent as that shown for the reporter gene from zinc-depleted wild type cells substantiated the conclusion that PAH1 expression was induced by a low cytosolic level of zinc (Fig. 3B). In addition, the zap1Δ mutation caused the attenuation of the induced expression of the P_PAH1-lacZ reporter gene in response to zinc depletion (Fig. 3B). This result substantiated the conclusion that Zap1p played a role in the zinc-mediated regulation of PAH1.

Zap1p Interacts with UAS_ZRE Sequences in PAH1 Promoter—The PAH1 promoter contains three regions (designated ZRE1, ZRE2, and ZRE3) that share 64% sequence homology with the consensus UAS_ZRE sequence (ACCTTNAAGGT) for Zap1p interaction (11) (Fig. 4A). To determine whether the putative ZRE sequences are Zap1p-binding sites, we performed electrophoretic mobility shift assays with oligonucleotide probes containing the putative UAS_ZRE sequences and purified GST-Zap1p (687–880) (47). Of the three probes, ZRE2 showed the strongest interaction with GST-Zap1p (687–880) (Fig. 4, B and C). The interaction with ZRE2 was 3- and 5-fold greater, respectively, when compared with the interactions with ZRE1 and ZRE3. The specificity of GST-Zap1p (687–880) binding to the three sequences was examined further using the same assay. The formation of the complexes was dependent on the concentration of GST-Zap1p (687–880) (Fig. 4B), and the unlabeled probes competed with the labeled probes for GST-Zap1p (687–880) binding in a concentration-dependent manner (Fig. 4C). To further examine the specificity of GST-Zap1p (687–880) for interactions with ZRE1, ZRE2, and ZRE3, the sequences were changed to the nonconsensus UAS_ZRE sequence of AAAAANAAAAA. These changes abolished the interactions with GST-Zap1p (687–880) (data not shown). These data supported the conclusion that the Zap1p-mediated regulation of PAH1 expression occurred by a mechanism that involves the direct interaction of Zap1p with the PAH1 promoter.

We confirmed that the induction of PAH1 in response to zinc deficiency was dependent on the ZRE2 sequence in the promoter. The ZRE2 sequence within the P_PAH1-lacZ reporter gene was mutated to AAAAAAAAAAA. Cells (wild type strain DY1457) bearing the mutant PAH1-lacZ plasmid were grown in the absence of 1.5 μM ZnSO4. Cell extracts were extracted and assayed for β-galactosidase activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D. (error bars). The differences in the β-galactosidase activities of the wild type controls in A and B were due to strain differences.

FIGURE 3. Depletion of cytosolic zinc causes Zap1p-mediated induction of P_PAH1-lacZ reporter gene activity. A, wild type (strain W303-1A) cells bearing the P_PAH1-lacZ reporter plasmid pFP1 were grown in the presence of the indicated concentrations of ZnSO4. B, wild type (strain DY1457), zrt1Δ zrt2Δ mutant (strain ZHY3), and zap1Δ mutant (strain ZHY6) cells bearing the P_PAH1-lacZ plasmid were grown in the absence and presence of 1.5 μM ZnSO4. Cell extracts were prepared and assayed for β-galactosidase activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D. (error bars). The differences in the β-galactosidase activities of the wild type controls in A and B were due to strain differences.
FIGURE 5. pah1Δ mutation causes derepression of PS synthase activity and ablates zinc-mediated regulation of enzyme. Wild type (strain W303-1A), dpp1Δ lpp1Δ mutant (strain TBY1), and pah1Δ mutant (strain GHY57) cells were grown in the absence and presence of 1.5 μM ZnSO4. Cell extracts were prepared and assayed for PS synthase activity. Each data point represents the average of triplicate enzyme determinations from two independent experiments ± S.D. (error bars).

As discussed above, PA phosphatase activity may provide DAG that is used for the synthesis of PC via the Kennedy pathway. When Cells Are Deficient of Zinc—Choline kinase, the enzyme that catalyzes the committed step in PC synthesis via the Kennedy pathway, is induced by zinc depletion through the interaction of Zap1p with UASZRE sequences in the CKI1 promoter (7) (Fig. 1B). Like PS synthase, the expression of choline kinase is governed by the regulatory circuit that includes the Ino2p-Ino4p activation complex, the Opi1p repressor, and the UASINO element in the CKI1 promoter (7, 15) (Fig. 1A). Because Opi1p repressor function is mediated by PA content, we questioned what effect the pah1Δ mutation would have on choline kinase expression and its regulation in response to zinc deficiency. For cells grown in zinc-replete medium, the choline kinase activity in the pah1Δ mutant was 1.75-fold higher than that found in wild type cells (Fig. 6A). As described previously (7), depletion of zinc from wild type cells resulted in the induced expression (2.6-fold) of choline kinase activity (Fig. 6A). For pah1Δ mutant cells, the depletion of zinc still caused the induction (2-fold) in choline kinase activity, and thus, for zinc-deficient cells, the activity in the pah1Δ mutant was elevated (1.4-fold) when compared with the wild type control (Fig. 6A).

As discussed above, PA phosphatase activity may provide DAG that is used for the synthesis of PC via the Kennedy pathway (19, 20) (Fig. 1). Because of the fact that PAH1 expression was induced by zinc deficiency, we questioned what effect the pah1Δ mutation would have on the zinc-mediated regulation of the pathway. Cells were labeled to steady state with [methyl-14C]choline followed by the extraction and analyses of the water-soluble Kennedy pathway intermediates and PC. As described previously (7), zinc depletion of wild type cells caused an increase in the label that was incorporated into phosphocholine (10-fold) and PC (2.5-fold) but no change in the amount of CDP-choline. Consistent with the induced expression of choline kinase activity, pah1Δ mutant cells exhibited elevated levels of phosphocholine when grown in zinc-replete (10-
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![Figure 6](image_url)

**FIGURE 6.** *pah1Δ* mutation causes derepression of choline kinase activity but inhibits synthesis of PC via Kennedy pathway when cells are deficient of zinc. Wild type (strain W303-1A) and *pah1Δ* mutant (strain GNY57) cells were grown in the absence and presence of 1.5 μM ZnSO₄. A, cell extracts were prepared and assayed for choline kinase activity. Each data point represents the average of triplicate enzyme determinations from two independent experiments. S.D. ± the cultures were incubated with [methyl-¹⁴C]choline (0.2 μCi/ml) to uniformly label the Kennedy pathway intermediates and PC. The water-soluble intermediates and PC were extracted and analyzed separately by TLC. The ¹⁴C-labeled compounds were visualized by phosphorimaging, and their amounts in cpm were determined from a standard curve using ImageQuant software. The values reported are the average of three separate experiments ± S.D. (error bars). Cho, choline; P-Cho, phosphocholine.

Neutral Lipid Composition Is Affected in *pah1Δ* Mutant Cells Deficient of Zinc—Because the Pah1p PA phosphatase was induced in response to zinc depletion, we questioned what effect the *pah1Δ* mutation would have on the TAG content of zinc-deficient cells. Wild type and *pah1Δ* mutant cells were labeled to steady state with [2-¹⁴C]acetate followed by the extraction and analysis of lipids by TLC (Fig. 7). For wild type cells, zinc depletion did not affect TAG content, but it did cause an increase (2.4-fold) in the content of ergosterol esters. As described previously for zinc-replete cells (18), the *pah1Δ* mutation caused a decrease in the levels of TAG (87%), DAG (20%), and ergosterol (49%) but caused an increase in the amount of ergosterol esters (100% decrease). The increased amount of ergosterol esters in the *pah1Δ* mutant caused a decrease in the levels of TAG (87%), DAG (20%), and ergosterol (49%) but caused an increase in the amount of ergosterol esters (100% decrease). The increased amount of ergosterol esters in the *pah1Δ* mutant caused a decrease in the levels of TAG (87%), DAG (20%), and ergosterol (49%) but caused an increase in the amount of ergosterol esters (100% decrease). The increased amount of ergosterol esters in the *pah1Δ* mutant caused a decrease in the levels of TAG (87%), DAG (20%), and ergosterol (49%) but caused an increase in the amount of ergosterol esters (100% decrease). The increased amount of ergosterol esters in the *pah1Δ* mutant caused a decrease in the levels of TAG (87%), DAG (20%), and ergosterol (49%) but caused an increase in the amount of ergosterol esters (100% decrease). The increased amount of ergosterol esters in the *pah1Δ* mutant caused a decrease in the levels of TAG (87%), DAG (20%), and ergosterol (49%) but caused an increase in the amount of ergosterol esters (100% decrease).

**DISCUSSION**

Zinc is an essential nutrient in *S. cerevisiae* and in higher eukaryotic organisms (1, 49). It serves as a cofactor for hundreds of enzymes and is a structural component of many proteins (49–51). Thus, the cellular concentrations of zinc must be tightly regulated (1, 52). Cellular zinc status is primarily controlled by plasma membrane zinc transporters (45, 46, 53) as well as by the zinc transporters found in the membranes of the vacuole (29, 54–56), endoplasmic reticulum (51, 57), and mitochondria (58). The expression of many of these transporters (e.g. plasma membrane-associated Zrt1p) is induced (via Zap1p) when zinc is deficient, and interestingly, this regulation is coordinated with changes in membrane phospholipid composition that are brought about by the transcriptional regulation of several phospholipid synthesis genes (1, 2, 52). Although the expression of some phospholipid synthesis genes (e.g. *PIS1*, *C2I3, and EKI1*) is induced (via Zap1p), some genes (e.g. *CHO1*) are repressed (via Opil1p) (2) (Fig. 1). This genetic regulation, coupled to biochemical mechanisms that occur through the availability of phospholipid synthesis substrates and intermediates, causes the PI content to decrease, the PE content to increase, and the PC content to remain unchanged (2, 4, 59). Stabilization of PC content under zinc-limiting conditions results from a balance between the inhibition of PC synthesis via the CDP-DAG pathway and the activation of PC synthesis via the Kennedy pathway (2, 4). In this work, we addressed the hypothesis that *PAH1*-encoded PA phosphatase activity plays an important role in the zinc-mediated regulation of phospholipid synthesis. The impetus for this work stems from the fact that the PA phosphatase enzyme controls cellular levels of PA (a precursor to both CDP-DAG and Kennedy pathways and a regulator of Opil1p repressor function) and may provide the
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The derepression of the CHO1-encoded PS synthase and the CKI1-encoded choline kinase activities in zinc-replete pah1Δ mutant cells was consistent with that observed for other UAS\textsubscript{INO}-containing genes (e.g. OPI3, INO1, and INO2) (21). The basis for the derepression of these genes is the attenuation of Opi1p repressor function due to elevated PA content caused by the pah1Δ mutation (4, 15, 22, 68). That the pah1Δ mutation eliminated the zinc-mediated regulation of PS synthase supported the notion that PA phosphatase activity controlled Opi1p repressor function. On the other hand, the pah1Δ mutation did not eliminate the zinc-mediated regulation of choline kinase, indicating that the repressive effect that Opi1p would have on expression was overcome by the derepression of CKI1 by Zap1p (7).

In addition to its role in regulating the PA-mediated control of Opi1p repressor function, the choline labeling studies confirmed that Pah1p PA phosphatase plays a role in PC synthesis via the Kennedy pathway. For wild type cells deficient of zinc, the increased levels of phosphocholine and PC were consistent with the inductions of both choline kinase and PA phosphatase activities. However, in pah1Δ mutant cells (that already had an elevated PC content with zinc), the depletion of zinc did not cause an increase in PC, but instead there was a decrease in PC, a result consistent with the loss of PA phosphatase activity. With respect to neutral lipids, the loss of Pah1p caused a dra-
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mamic decrease in TAG (87%) content in zinc-replete cells (18). However, the level of TAG in pah1Δ mutant cells increased by over 3-fold when zinc was removed from the growth medium. This result was consistent with the induction of the PA phosphatase activity encoded by the unknown gene(s). However, this interpretation, as well as any conclusions about the role this other PA phosphatase enzyme plays in PC synthesis, cannot be confirmed until its gene(s) is identified and a mutant becomes available for analysis.

A question that has yet to be resolved is why phospholipid composition is regulated in response to zinc deficiency. Although many genes in cellular metabolism are either induced or repressed depending on zinc status, the genes that are most highly regulated are those encoding zinc transporters (1, 67). Thus, changes in membrane phospholipid composition in response to zinc might be important to the structure and/or function of the zinc transporters that are embedded in the membrane bilayer. The availability of mutants defective in the regulation and/or synthesis of membrane phospholipids should facilitate defined studies on the importance of membrane phospholipid composition to zinc transport function in S. cerevisiae.

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