Ethanalamine kinase catalyzes the committed step in the synthesis of phosphatidylethanalamine via the CDP-ethanalamine branch of the Kennedy pathway. Regulation of the EKI1-encoded ethanalamine kinase by the essential nutrient zinc was examined in Saccharomyces cerevisiae. The level of ethanalamine kinase activity increased when zinc was depleted from the growth medium. This regulation correlated with increases in the CDP-ethanalamine pathway intermediates phosphoethanolamine and CDP-ethanalamine, and an increase in the methylated derivative of phosphatidylethanalamine, phosphatidylcholine. The β-galactosidase activity driven by the P_{EKI1}-lacZ reporter gene was elevated in zinc-depleted cells, indicating that the increase in ethanalamine kinase activity was attributed to a transcriptional mechanism. The expression level of P_{EKI1}-lacZ reporter gene activity in the zrt1Δzrt2Δ mutant (defective in plasma membrane zinc transport) cells grown with zinc was similar to the activity expressed in wild-type cells grown without zinc. This indicated that EKI1 expression was sensitive to intracellular zinc. The zinc-mediated regulation of EKI1 expression was attenuated in the zap1Δ mutant defective in the zinc-regulated transcription factor Zap1p. Direct interactions between Zap1p and putative zinc-responsive elements in the EKI1 promoter were demonstrated by electrophoretic mobility shift assays. Mutations of these elements to a nonconsensus sequence abolished Zap1p-DNA interactions. Taken together, this work demonstrated that the zinc-mediated regulation of ethanalamine kinase and the synthesis of phospholipids via the CDP-ethanalamine branch of the Kennedy pathway were controlled in part by Zap1p.

The yeast Saccharomyces cerevisiae responds to a variety of stress conditions (e.g. nutrient depletion) by regulating the expression of several enzyme activities including those involved in phospholipid synthesis (1–6). In S. cerevisiae, the major membrane phospholipids phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are synthesized by complementary (CDP-diacylglycerol and Kennedy) pathways (Fig. 1), and these pathways are regulated by genetic and biochemical mechanisms (6–11). The phospholipid biosynthetic enzymes are presumably regulated to control membrane phospholipid composition. Phospholipids govern many membrane-associated functions including enzyme catalysis, receptor-mediated signaling, and solute transport (12, 13). In addition, phospholipids are precursors for the synthesis of macromolecules (14–18), serve as molecular chaperons (19, 20), serve in protein modification for membrane association (21), and are reservoirs of second messengers (22).

Zinc is an essential nutrient required for the growth and metabolism of S. cerevisiae, and of higher eukaryotes (23). The essential nature of zinc stems from the role it plays as a cofactor for hundreds of enzymes and from its role as a structural constituent of some proteins (23–25). Nonetheless, zinc is toxic to cells when accumulated in excess amounts (23). In S. cerevisiae, the cellular levels of zinc are largely controlled by plasma membrane zinc transporters (Zrt1p, Zrt2p, Fet4p) (26–28) and by zinc transporters found in the membranes of the vacuole (Zrt3p, Cot1p, Zrc1p) (29–32), endoplasmic reticulum (Msc2p, Zrg17p), (25, 33), and mitochondria (Mrs3p, Mrs4p) (34). Most of these zinc transporters are regulated at the transcriptional level to maintain zinc homeostasis (35). For example, when the cellular level of zinc is limiting, the expression of the high affinity zinc transporter Zrt1p is induced for increased zinc uptake, but when the cellular level of zinc is high, the expression of Zrt1p is repressed to attenuate zinc uptake (26). The increase in Zrt1p expression is dependent on the transcription factor Zap1p, which interacts with a UAS_{Zap1} in the promoter of the ZRT1 gene to activate transcription (26, 36, 37). In addition, when the cellular level of zinc is limiting, Zrt1p is a stable protein (38), but when the level of zinc is high, Zrt1p is ubiquitinated (39) and removed from the plasma membrane by endocytosis and vacuolar degradation (38).

Interestingly, phospholipid metabolism is regulated by the cellular level of zinc in S. cerevisiae (4, 5, 40). In fact, the DPP1 gene, which encodes the vacuole membrane-associated diacylglycerol pyrophosphate phosphatase enzyme, is one of the most highly regulated Zap1p targets that respond to zinc depletion in the S. cerevisiae genome (41, 42). The induction of diacylglycerol pyrophosphate phosphatase expression in zinc-depleted cells results in reduced levels of the minor vacuole membrane phospholipid diacylglycerol pyrophosphate and phosphatidate (40). Moreover, the cellular level of zinc regulates the synthesis of the major membrane phospholipids in S. cerevisiae (4). The activity levels of the CDP-diacylglycerol pathway enzymes PS synthase, PS decarboxylase, and the phospholipid methyltransferases are reduced in zinc-depleted cells (4). In contrast, the activity of the CDP-diacylglycerol branch point enzyme PI synthase is elevated in response to zinc depletion (4, 43). For the PS synthase enzyme, the repression of CHO1 transcription is mediated by the phospholipid synthesis transcription factor Opi1p (4). For the PI synthase enzyme, the induction of PIS1 transcription is mediated by Zap1p (43). The induction of PI synthase activity correlates with an increase in PI content, whereas the repression of PS synthase and PS decarboxylase activities corre-

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2 The abbreviations used are: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; UAS_{Zap1}, upstream activating sequence zinc-responsive element; UAS_{non}, upstream activating sequence inositol-responsive element; WT, wild type; GST, glutathione S-transferase.

3 The diacylglycerol pyrophosphate phosphatase enzyme catalyzes the dephosphorylation of the β-phosphate from diacylglycerol pyrophosphate to form phosphatidate, and then removes the phosphate from phosphatidate to form diacylglycerol (68).
late with a decrease in PE content (4). Although the activities of the phospholipid methyltransferases (that methylate PE to form PC) are repressed in zinc-depleted cells, this growth condition does not have a major effect on PC content (4).

In this work, we examined the contribution of the CDP-ethanolamine branch of the Kennedy pathway for the reduction in PE content in response to zinc depletion. We focused on the regulation of the EKI1-encoded ethanolamine kinase, the enzyme that catalyzes the committed step in the CDP-ethanolamine pathway. Unexpectedly, we found that the expression of ethanolamine kinase activity, and the CDP-ethanolamine pathway was induced upon zinc depletion. In addition, this growth condition resulted in an increase in PC derived from PE synthesized via the CDP-ethanolamine pathway. The induction of ethanolamine kinase activity was attributed to a transcriptional mechanism that was mediated in part by the Zap1p transcription factor.

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. The Yeastmaker yeast transformation kit was obtained from Clontech. Oligonucleotides for electrophoretic mobility shift assays were prepared by Genosys Biotechnology, Inc. ProbeQuant G-50 columns were purchased from Amersham Biosciences. Protein molecular mass standards for SDS-PAGE, protein assay reagents, electrophoretic reagents, and acrylamide solutions were purchased from Bio-Rad. Ampicillin, aprotinin, benzamidine, bovine serum albumin, ethanolamine, phosphoethanolamine, CDP-ethanolamine, leupeptin, O-nitrophenyl β-D-galactopyranoside, pepstatin, phenylmethylsulfonyl fluoride, and IGEPAL CA-630 were purchased from Sigma. Phospholipids were purchased from Avanti Polar Lipids. Radiochemicals and scintillation counting supplies were purchased from PerkinElmer Life Sciences and National Diagnostics, respectively. Liqui-Nox detergent was from Alconox, Inc. Silica gel 60 thin layer chromatography plates were from EM Science.

Strains, Plasmids, and Growth Conditions—The strains and plasmids used in this work are listed in Table 1. Transformation of yeast (44, 45) and bacteria (46) were performed as described previously. Yeast cultures were grown in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or in synthetic complete medium (47) containing 2% glucose at 30 °C. The appropriate amino acids of synthetic complete medium were omitted for selection purposes. Zinc-free medium was synthetic complete medium (47) prepared with yeast nitrogen base lacking zinc sulfate. For zinc-depleted cultures, cells were first grown for 24 h in synthetic complete medium containing 1.5 μM zinc sulfate (equivalent to the concentration of zinc in standard synthetic growth media). Saturated cultures were diluted into zinc-free medium at an initial concentration of 1×10⁶ cells/ml, and grown for 24 h. Cultures were then diluted to 1×10⁶ cells/ml and grown in zinc-free medium containing 0 or 1.5 μM zinc sulfate. This growth routine was used to deplete internal stores of zinc (5). Plasmid maintenance and amplification were performed in Escherichia coli strain DH5α. 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. For growth on plates, yeast and bacterial media were supplemented with 2% and 1.5% agar, respectively. Yeast cell numbers in liquid medium were determined spectrophotometrically at an absorbance of 600 nm. Exponential phase cells were harvested at a density of 1×10⁷ 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.

Preparation of Cell Extracts and Protein Determination—All steps were performed at 0 °C. Yeast cells were disrupted with glass beads with a Mini-BeadBeater-8 (Biospec Products) in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 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 (48). Glass beads and cell debris were removed by centrifugation at 1,500 × g for 10 min, and the supernatant was used as the cell extract. The concentration of protein in cell extracts was estimated by the method of Bradford (49) using bovine serum albumin as the standard.

Enzyme Assays—Ethanolamine kinase activity was measured for 40 min at 30 °C by following the phosphorylation of [1,2-14C]ethanolamine (20,000 cpm/nmol) with ATP. The reaction mixture contained 50 mM

![FIGURE 1. Phospholipid synthesis in S. cerevisiae. The pathways shown for the synthesis of phospholipids include the relevant steps discussed throughout the article. The genes encoding enzymes responsible for the reactions in the pathways are indicated in the figure. The reaction catalyzed by the EKI1-encoded ethanolamine kinase enzyme is highlighted in the box. CDP-DAG, CDP-diacylglycerol; P-choline, phosphocholine; PA, phosphatidate.](image)

TABLE 1

| Strain or plasmid | Genotype or relevant characteristics | Source or Ref. |
|-------------------|-------------------------------------|---------------|
| **E. coli** | DH5α | F− d800lacZ::M15 ΔlacZ15::argF)U169 deoR recA1 endA1 hsdR17(rK− m−) pLOA supE44 thi-1 gyrA96 relA1 | 46 |
| **S. cerevisiae** | W303–1A | MATA ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 | 69 |
| | DY1457 | MATA ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52 | 37 |
| | ZY16 | MATA ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52 | 37 |
| | ZY13 | MATA ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52 trp1-12 leu3-112 trp1-1 fpr1 Δ trp1-1 fpr1 Δ | 27 |
| | SH303 | MATA his3 Δ200 leu2-Δ1 Δ trp1-1 Δ ura3-52 ino2a Δ trp1 | S. A. Henry |
| | SH307 | MATA his3 Δ200 leu2-Δ1 Δ trp1-1 Δ ura3-52 ino4Δ Δ LEU2 | S. A. Henry |
| | SH304 | MATA his3 Δ200 leu2-Δ1 Δ trp1-1 Δ ura3-52 ino4Δ Δ LEU2 | S. A. Henry |
| **Plasmid** | pR5K10 | P_EKI1::lacZ reporter gene containing the EKI1 promoter with LEU3 | 54 |
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Tris-HCl buffer, pH 8.5, 5 mM ethanolamine, 10 mM ATP, 10 mM MgSO4, and enzyme protein (0.12 mg/ml) in a final volume of 25 μl. Reaction mixtures were separated by thin layer chromatography on potassium oxalate-impregnated silica gel plates using the solvent system of methanol/0.6% sodium chloride/29.2% ammonium hydroxide (10:10:1) to determine the positions of the labeled compounds on chromatograms. The levels of ethanolamine, phosphoethanolamine, and CDP-ethanolamine in the labeling period were analyzed by thin layer chromatography. The levels of ethanolamine and phosphoethanolamine, and CDP-ethanolamine in the labeling period were determined by phosphorimaging and compared with standards. The amount of labeled product was determined by scintillation counting. β-Galactosidase activity was determined by measuring the conversion of O-nitrophenyl β-d-galactopyranoside to O-nitrophenol (molar extinction coefficient of 3500 M⁻¹ cm⁻¹) by following the increase in absorbance at 410 nm on a recording spectrophotometer (51). The reaction mixture contained 100 mM sodium phosphate buffer, pH 7.0, 3 mM O-nitrophenyl β-d-galactopyranoside, 1 mM MgCl2, 100 mM 2-mercaptoethanol, and enzyme protein in a total volume of 0.1 ml. A unit of ethanolamine kinase activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product/min. A unit of β-galactosidase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol product/min. All assays were performed in triplicate and were linear with time and protein concentration. Specific activity was defined as units per mg of protein.

Labeling and Analysis of CDP-ethanolamine Pathway Intermediates and Phospholipids—Exponential phase cells were labeled for five to six generations with [1,2-14C]ethanolamine (0.5 μCi/ml). The CDP-ethanolamine pathway intermediates and phospholipids were extracted from whole cells by a chloroform/methanol/water extraction, followed by the separation of the aqueous and chloroform phases (52). The aqueous phase was neutralized, dried in vacuo, and the residue was dissolved in deionized water. Samples were subjected to centrifugation at 12,000 × g for 3 min to remove insoluble material. The 14C-labeled CDP-ethanolamine pathway intermediates were then separated by thin-layer chromatography on silica gel plates using the solvent system methanol/0.6% sodium chloride/ammonium hydroxide (10:10:1, v/v). 14C-labeled phospholipids, which were contained in the chloroform phase, were analyzed by thin-layer chromatography on silica gel plates using the solvent system methanol/0.6% sodium chloride/ammonium hydroxide (10:10:1, v/v). The positions of the labeled compounds on chromatograms were determined by phosphorimaging and compared with standards. The amount of each labeled compound was determined by liquid scintillation counting.

Electrophoretic Mobility Shift Assays—The double-stranded oligonucleotides used in the electrophoretic mobility shift assays were presented in Table 2. They were prepared by annealing 25 μM complementary single-stranded oligonucleotides in a reaction mixture (0.1 ml) containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA. The annealing reactions were incubated for 5 min at 100 °C in a heat block, and then kept in the heat block for another 2 h after it had been turned off. The annealed oligonucleotides (100 pmol), which had a 5′ overhanging end, were labeled with [γ-32P]dATP (400–800 Ci/nmol) and Klenow fragment (5 units) for 30 min at room temperature. Labeled oligonucleotides were separated from unincorporated nucleotides by gel filtration using ProbeQuant G-50 spin columns.

GST-Zap1p was expressed and purified from E. coli (43). The indicated amounts of GST-Zap1p were incubated with 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, CA-630, 10% glycerol, and 1 pmol of radiolabeled DNA probe (2.5 × 104 cpm pmol) for 15 min at room temperature in a total volume of 10 μl. The reaction mixtures were resolved on 6% polyacrylamide gels (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.

Data Analysis—Statistical significance was determined by performing the Student’s t test using SigmaPlot software. p values < 0.05 were taken as a significant difference.

RESULTS

Effect of Zinc Depletion on Ethanolamine Kinase Activity and on the Incorporation of Ethanolamine into CDP-ethanolamine Pathway Intermediates and Phospholipids—The effect of zinc depletion on ethanolamine kinase activity was examined. For this and subsequent experiments, the growth medium lacked inositol supplementation to preclude the regulatory effects that inositol has on the regulation of phospholipid synthesis (6–9, 53). Depletion of zinc from the growth medium of wild-type cells caused a 2-fold increase in ethanolamine kinase activity when compared with cells grown in the absence of zinc (Fig. 2). To examine the effects of zinc depletion on the synthesis of PE via ethanolamine kinase and the CDP-ethanolamine branch of the Kennedy pathway, wild-type cells that were grown in the absence and presence of 1.5 μM zinc. Cell extracts were prepared and assayed for ethanolamine kinase activity. Each data point represents the average of triplicate determinations from two independent experiments ± S.D.

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Table 2: Oligonucleotides used for electrophoretic mobility shift assays

| Element | Annealed oligonucleotides* |
|---------|----------------------------|
| EKI1 ZRE1 | 5'-ATCATATACCTTCAGAATTGCA-3' |
| EKI1 ZRE2 | 5'-ATCCGCTCTCCTTTAGACAAAT-3' |
| EKI1 ZRE3 | 5'-GTAAAAAAATATCCGTTGCTGCT-3' |
| EKI1 ZRE1 (Mt) | 5'-ATCATATACCTTCAGAATTGCA-3' |
| EKI1 ZRE3 (Mt) | 5'-GTAAAAAAATATCCGTTGCTGCT-3' |
| ZRE1 Consensus (C) | 5'-GTAAAAAAATATCCGTTGCTGCT-3' |
| ZRE3 Consensus (C) | 5'-GTAAAAAAATATCCGTTGCTGCT-3' |

* Underlined sequences are putative ZRE sites. The mutations (Mt and C) in ZRE1 and ZRE3 are shown in bold letters. The lower case letters indicate the nucleotides filled with the Klenow fragment.
Effect of Zinc Depletion on the Expression of EKI1—We examined the effect of zinc depletion on the expression of the EKI1 gene. This analysis was facilitated by use of a P_{EKI1} lacZ reporter gene where the expression of β-galactosidase activity is dependent on transcription driven by the EKI1 promoter (54). Wild-type cells bearing the P_{EKI1} lacZ reporter gene were grown to the exponential phase of growth in the absence or presence of various concentrations of zinc. Cell extracts were then prepared and used for the assay of β-galactosidase activity. The depletion of zinc from the growth medium resulted in a concentration-dependent increase in β-galactosidase activity (Fig. 4). Based on this assay, the expression of EKI1 in zinc-depleted cells was 8-fold greater than the expression found in cells grown with 1.5 μM zinc. Concentrations of zinc that were greater than 1.5 μM did not have a significant effect on the expression of β-galactosidase activity (Fig. 4). The induced level (8-fold) of reporter gene activity was greater than the induced level (2-fold) of ethanolamine kinase activity. This discrepancy is not uncommon because lacZ fusion proteins are generally stable and not subject to turnover (55).

To address whether the zinc-mediated regulation of EKI1 expression was caused by the extracellular or intracellular levels of zinc, a P_{EKI1} lacZ reporter activity was examined in zrt1 Δ zrt2Δ mutant cells grown in the presence of zinc. The zrt1 Δ zrt2Δ mutant is defective in both the high affinity (Zrt1p) and low affinity (Zrt2p) plasma membrane zinc transporters and has a low intracellular level of zinc (26, 27). Mutant cells grown in the presence of zinc exhibited a high level of β-galactosidase activity that was similar to that shown for wild-type cells grown in the absence of zinc (Fig. 5). This indicated that EKI1 expression was governed by the intracellular level of zinc.

Effects of the ino1Δ, ino4Δ, and opi1Δ Mutations on the Zinc-mediated Regulation of EKI1 Expression—Previous work has shown that enzymes responsible for PE synthesis via the CDP-diacylglycerol pathway are repressed in response to zinc depletion (4). The zinc-mediated repression of one of these enzymes (i.e. CHO1-encoded phosphatidylserine synthase) is mediated by the negative phospholipid synthesis transcription factor Opi1p (4). Opi1p represses transcription by binding to the positive transcription factor Ino2p that exists in a complex with another positive transcription factor Ino4p (56). The Ino2p/Ino4p complex interacts with a UASINO element in the gene promoter to activate transcription (6, 8, 9). Because the EKI1 gene contains a UASINO element and its transcription is controlled by Ino2p, Ino4p, and Opi1p (54), we questioned whether these transcription factors played a role in the zinc-mediated regulation of EKI1 expression. P_{EKI1} lacZ reporter gene activity was measured in ino1Δ, ino4Δ, and opi1Δ mutant cells grown in the absence or presence of zinc. The β-galactosidase activity found in these mutants was not affected by zinc depletion (data not shown). This indicated that Ino2p, Ino4p, and Opi1p were not involved with the zinc-mediated regulation of EKI1.

Effect of the zap1Δ Mutation on the Zinc-mediated Regulation of EKI1 Expression—The induction of the DPP1-encoded diacylglycerol pyrophosphate phosphatase and the PIS1-encoded phosphatidylinositol synthase in response to zinc depletion is mediated by the zinc-regulated positive transcription factor Zap1p (5, 43). Zap1p, which itself is induced by zinc depletion (37), interacts with a UASZRE in the promoters of DPP1 and PIS1 to activate transcription when cells are depleted for zinc (5, 43). As discussed below, the EKI1 promoter contains putative UASZRE sequences, and thus we examined whether the zinc-mediated regulation of EKI1 was dependent on Zap1p function. P_{EKI1} lacZ reporter gene activity was induced in zap1Δ mutant cells grown without zinc. However, the level of induction was attenuated (~50%) when compared with the regulation found in the wild-type control (Fig. 6). This result indicated that Zap1p played a role in the zinc-mediated regulation of EKI1 expression.

Binding of Zap1p to Putative ZRE Sequences in the EKI1 Promoter—The EKI1 promoter contains three regions (ZRE1, ZRE2, ZRE3) with sequence homology to the consensus UASZRE sequence (ACCTTACCTT). Recombinant GST-Zap1p687–880 was purified from E. coli. Zap1p687–880 contains the UASZRE binding domain (amino acids 687–880 of Zap1p) (57). Of the three probes, the oligonucleotide containing ZRE1 showed the strongest interaction with GST-
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Zap1p<sub>687–880</sub> (Fig. 7B). The interaction of GST-Zap1p<sup>687–880</sup> with ZRE3 was about 3-fold lower compared with ZRE1, whereas an interaction with ZRE2 was not detectable (Fig. 7B). The specificity of GST-Zap1p<sup>687–880</sup> binding to ZRE1 and ZRE3 was examined further using nonconsensus UASZRE sequence. These mutations abolished the interaction with GST-Zap1p<sup>687–880</sup> (Fig. 9, A and B). The consensus UASZRE sequence of the most highly regulated Zap1p target genes is 5′-ACCTTGAAGGT-3′ (36, 41). For comparison of GST-Zap1p<sup>687–880</sup> binding to ZRE1 and ZRE3, their sequences were mutated to a consensus UASZRE sequence. The interaction of GST-Zap1p<sup>687–880</sup> with the consensus sequence was 11- and 30-fold greater, respectively, when compared with interactions to the wild-type ZRE1 and ZRE3 sequences (Fig. 9, A and B).

FIGURE 6. Effect of zap1Δ mutation on the regulation of the EKI1 gene by zinc depletion. WT and zap1Δ mutant cells bearing the P<sub>Zap1</sub>-lacZ reporter plasmid pKSK10 were grown in the absence and presence of 1.5 μM zinc. Cell extracts were prepared and assayed for β-galactosidase activity. Each data point represents the average of triplicate determinations from two independent experiments ± S.D.

FIGURE 7. Interactions of GST-Zap1p<sup>687–880</sup> with putative ZRE sequences in the EKI1 promoter. A, the locations and sequences of the putative ZRE sites in the EKI1 promoter are shown in the figure. B, samples (1 pmol) of radiolabeled double-stranded synthetic oligonucleotides (2.5 × 10<sup>5</sup> cpm/pmol) with sequences for ZRE1 (lane 1) 5′-ACCTTTGCAAGA-3′, ZRE2 (lane 2) 5′-TCCTTGAAGAC-3′, and ZRE3 (lane 3) 5′-ATCGTTTGTTG-3′, the EKI1 promoter were incubated with 0.5 μg of recombinant GST-Zap1p<sup>687–880</sup>. Interaction of GST-Zap1p<sup>687–880</sup> with the labeled oligonucleotides was determined by electrophoretic mobility shift assay using a 6% polyacrylamide gel. The data shown are representative of two independent experiments.

FIGURE 8. Concentration dependences of Zap1p<sup>687–880</sup> interactions with ZRE1 and ZRE3 in the EKI1 promoter. Samples (1 pmol) of radiolabeled double-stranded synthetic oligonucleotides (2.5 × 10<sup>5</sup> cpm/pmol) with sequences for ZRE1 and ZRE3 in the EKI1 promoter were incubated with recombinant GST-Zap1p<sup>687–880</sup> A and C, the experiment was performed with 0, 0.15, 0.3, and 0.5 μg of recombinant GST-Zap1p<sup>687–880</sup>, B and D, the experiment was performed with 0, 25, 50, and 100 pmol of unlabeled oligonucleotide with the sequences for ZRE1 and ZRE3, respectively. Interaction of GST-Zap1p<sup>687–880</sup> with the labeled oligonucleotides was determined by electrophoretic mobility shift assay using a 6% polyacrylamide gel. The data shown are representative of two independent experiments.

FIGURE 9. Effect of mutations in ZRE1 and ZRE3 on interactions with Zap1p<sup>687–880</sup>. Samples (1 pmol) of radiolabeled double-stranded synthetic oligonucleotides (2.5 × 10<sup>5</sup> cpm/pmol) with sequences for WT and mutated forms of ZRE1 and ZRE3 were incubated with recombinant GST-Zap1p<sup>687–880</sup>. A, the wild-type ZRE1 sequence was mutated from 5′-ACCTTTGCAAGA-3′ to the nonconsensus sequence 5′-GTTCGGCAAGA-3′ (Mt). The concentration of GST-Zap1p<sup>687–880</sup> was increased from 0 to 100 pmol of unlabeled oligonucleotide with the sequences for ZRE1 and ZRE3, respectively. Interaction of GST-Zap1p<sup>687–880</sup> with the labeled oligonucleotides was determined by electrophoretic mobility shift assay using a 6% polyacrylamide gel. The data shown are representative of two independent experiments.

DISCUSSION

Regulation of phospholipid synthesis in S. cerevisiae is complex. The most abundant phospholipids PE and PC are synthesized by alternative CDP-diacylglycerol and Kennedy pathways, and the genes and enzymes in these pathways are regulated by transcriptional and biochemical mechanisms (6–11). The expression of many phospholipid synthesis genes is controlled by availability of the essential mineral zinc (4, 40). Zinc depletion leads to an increase in PI content and a decrease in PE increase in PI synthase activity, and decreased activities of the CDP-diacylglycerol and Kennedy pathways, and the genes and enzymes in these pathways are regulated by transcriptional and biochemical mechanisms (6–11). The expression of many phospholipid synthesis genes is controlled by availability of the essential mineral zinc (4, 40). Zinc depletion leads to an increase in PI content and a decrease in PE increase in PI synthase activity, and decreased activities of the CDP-diacylglycerol pathway enzymes PS synthase and PS decarboxylase (4, 43). In the vacuole membrane, zinc depletion also causes decreased amounts of the minor phospholipids phosphatidate and diacylglycerol pyrophosphate (40). These changes are attributed to increased expres-
sion of the vacuole-associated diacylglycerol pyrophosphate phosphatase enzyme (5, 40).

In this work, we questioned whether the CDP-ethanolamine branch of the Kennedy pathway played a role in the zinc-mediated regulation of PE content in cellular membranes. Our studies focused on the EKI1-encoded ethanolamine kinase, the first enzyme in the pathway. In contrast to the CDP-diacylglycerol pathway enzyme activities that are reduced in zinc-depleted cells (4), ethanolamine kinase activity was elevated (2-fold). In addition, the increased ethanolamine kinase activity correlated with an increase (2-fold) in the incorporation of ethanolamine into its reaction product phosphoethanolamine. Ethanolamine (1.8-fold), the ethanolamine kinase substrate, and CDP-ethanolamine (3.5-fold), the reaction product of the next enzyme (CTP-phosphoethanolamine cytidylytransferase) in the pathway, were also elevated in zinc-depleted cells. Thus, the elevated levels of all three intermediates would favor an increased flux through the pathway under this growth condition. Mechanisms responsible for the elevated levels of ethanolamine and CDP-ethanolamine were not addressed here. Interestingly, the steady-state level of PE synthesized via the CDP-ethanolamine pathway was not affected by zinc depletion. The PE synthesized via the CDP-ethanolamine pathway was utilized by the phospholipid methyltransferase enzymes of the CDP-diacylglycerol pathway to synthesize PC, and the amount of PC made through this route was elevated 2-fold in response to zinc depletion. The elevation of the PC synthesized via this route provides an explanation as to why the overall PC content, as determined by $^{32}$P$_i$ labeling, is not affected by zinc depletion despite the fact that this growth condition represses the phospholipid methyltransferase activities of the CDP-diacylglycerol pathway (4). Thus, the reduction of PE synthesized via the CDP-diacylglycerol pathway was compensated by the increase in PE synthesized by the CDP-ethanolamine pathway for the ultimate synthesis of PC. Preliminary studies indicate that PC synthesis via the CDP-choline branch of the Kennedy pathway is also activated when zinc is depleted from the growth medium. 4

Analysis of $^{\text{P}}$EXKI$\text{-lacz}$ reporter gene activity showed that the induction of ethanolamine kinase activity by reduced zinc was due to a transcriptional mechanism. It was technically difficult to measure and quantify changes in EKI1 mRNA and ethanolamine kinase protein levels in response to zinc depletion due to the low level of EKI1 expression in S. cerevisiae. The analysis of $^{\text{P}}$EXKI$\text{-lacz}$ reporter gene activity in the zrt1Δzrt2Δ double mutant defective in the plasma membrane zinc transporters Zrt1p and Zrt2p (26, 27) indicated that a decrease in the intracellular level of zinc was responsible for the induction of EKI1 expression. Previous studies have shown that the intracellular level of zinc is responsible for regulation of phospholipid composition in zinc-depleted cells (4). Zap1p, a positive transcription factor that is maximally expressed in cells depleted for zinc and repressed in cells grown with excess zinc (37), is responsible for the zinc-mediated induction of the DPP1 (5) and PIS1 (43) genes encoding diacylglycerol pyrophosphate phosphatase and PI synthase, respectively. The zinc-mediated induction of the $^{\text{P}}$EXKI$\text{-lacz}$ reporter gene was attenuated by 50% in zap1Δ mutant cells. This indicated that the regulation of EKI1 gene expression by zinc was mediated in part by the Zap1p transcription factor. That regulation by zinc depletion was not totally lost in the zap1Δ mutant indicated that additional transcription factors were involved in the regulation of EKI1 by zinc. The transcription factors Ino2p, Ino4p, and Opi1p, which play a role in the zinc-mediated regulation of CHO1 and INO1 (4), were not involved in the zinc-mediated regulation of EKI1 expression. Additional studies will be required to identify the other transcription factors involved in this regulation.

Zap1p mediates induction of the phospholipid metabolic genes DPP1 and PIS1 by binding to UASZRE sequences in their promoters (5, 41, 43). The EKI1 promoter contains three regions (ZRE1, ZRE2, ZRE3) with sequence homology to the consensus UASZRE found in the promoters of highly regulated Zap1p target genes (e.g. ZRT1, ZRT2, ZRT3, DPP1) (41). Electrophoretic mobility shift assays performed with DNA probes containing these sequences and purified GST-Zap1p$^{687-880}$ indicated that Zap1p interacted with ZRE1 and ZRE3. Interaction of GST-Zap1p$^{687-880}$ with ZRE1 was about 3-fold greater when compared with the interaction with ZRE3. These interactions were specific as indicated by dose-response curves of the interactions, and the loss of interactions when ZRE1 and ZRE3 were mutated to the nonconsensus UASZRE sequence. When ZRE1 and ZRE3 were mutated to the consensus UASZRE sequence, the interactions with GST-Zap1p$^{687-880}$ were greatly enhanced. This observation provides an explanation as to why the magnitude of induced ethanolamine kinase activity (2-fold) is considerably less than the induced level (~10-fold) of DPP1-encoded diacylglycerol pyrophosphate phosphatase activity. In addition, the relatively weak interactions of GST-Zap1p$^{687-880}$ with ZRE1 and ZRE3 when compared with the interaction with the consensus UASZRE sequence found in the DPP1 promoter explains why the EKI1 gene was not identified as a Zap1p target in a genome-wide cDNA microarray analysis of genes induced by zinc deprivation (41). Nevertheless, the 2-fold induction of ethanolamine kinase activity in response to zinc deprivation resulted in concomitant increases in the levels of CDP-ethanolamine pathway intermediates phosphoethanolamine and CDP-ethanolamine, and ultimately the PC derived from PE synthesized via this pathway.

Ethanolamine kinase, along with other phospholipid metabolic enzymes (4, 5, 43), was regulated by zinc depletion, and this regulation affected membrane phospholipid composition. The composition of membrane phospholipids affects numerous cellular processes including membrane transport (12, 13). Interestingly, the regulation of phospholipid metabolism occurs in a coordinate manner with several zinc transporters (26, 27, 29, 35). For example, zinc depletion results in the Zap1p-mediated induction of plasma membrane (Zrt1p, Zrt2p, Fet4p) and vacuole membrane (Zrt3p) zinc transporters to increase cytoplasmic levels of zinc (26, 27, 29, 35). The fact that the zinc transporters are located within the phospholipid bilayer of cellular membranes raises the question as to whether changes in phospholipid composition in response to reduced levels of zinc might regulate their function. In this regard, the PE content of the membrane is crucial to proper function of the lactose permease transporter of E. coli (60). PE content regulates lactose permease function by directing its assembly and stability within the membrane bilayer (12, 19, 20, 60–62). Availability of mutants (e.g. eki1Δ) defective in PE synthesis should facilitate studies to address the importance of PE content for zinc transport function in S. cerevisiae.

Alternative explanations for the zinc-mediated regulation in PE content stem from the roles this phospholipid plays in modifying proteins for their association to the membrane. For example, PE is used directly for covalent modification and membrane attachment of Agp8p, a protein essential to the process of autophagy (63–66). Autophagy is the bulk import of cytosolic components into the vacuole for degradation that occurs in cells because of nutrient stress (66). Indeed, zinc depletion results in an elevation of Agp8p-PE (4). PE is also used for the glycosylphatidylinositol modification of proteins for membrane attachment (15). The glycosylphatidylinositol anchor is attached to proteins through the amine group of phosphoethanolamine that is derived from PE (15). Interestingly, the MCD4 gene that encodes one of the enzymes responsible for the transfer of the phosphoethanolamine moi-

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4 A. Soto and G. M. Carman, unpublished data.
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ety of PE to make the anchor (67) is induced by zinc depletion (41). The importance of PE for Apg8p modification and for glycosylphosphatidylinositol anchor synthesis in response to zinc depletion warrants further examination.

In summary, this work showed that the intracellular level of zinc regulated the expression of the EKI1-encoded ethanolamine kinase of Saccharomyces cerevisiae. The induction of ethanolamine kinase activity in response to zinc depletion correlated with an increase in phospholipid synthesis via the CDP-ethanolamine branch of the Kennedy pathway. A transcriptional mechanism was responsible for the induction of ethanolamine kinase activity, and the Zap1p transcription factor played a role in this regulation. This work advances our understanding of the regulation of phospholipid synthesis by zinc and the transcriptional control of the EKI1 gene.

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REFERENCES
1. Becker, G. W., and Lester, R. L. (1977) J. Biol. Chem. 252, 8684 – 8691
2. Gricar, P., and Henry, S. A. (1999) Nucleic Acids Res. 27, 2043 – 2050
3. Homann, M. J., Poole, M. A., Gaynor, P. M., Hao, C.-T., and Carman, G. M. (1987) J. Bacteriol. 169, 533 – 539
4. Iwanyshyn, W. M., Han, G. S., and Carman, G. M. (2004) J. Biol. Chem. 279, 21976 – 21983
5. Han, G.-S., Johnston, C. N., Chen, X., Atenstaedt, K., Daum, G., and Carman, G. M. (2001) J. Biol. Chem. 276, 10126 – 10133
6. Carman, G. M., and Henry, S. A. (1999) Prog. Lipid Res. 38, 361 – 399
7. Carman, G. M., and Henry, S. A. (1989) Annu. Rev. Biochem. 58, 655 – 669
8. Greenberg, M. L., and Lopes, J. M. (1996) J. Biol. Chem. 271, 408 – 412
9. Hwang, K. L., and Patton-Vogt, J. L. (1998) Prog. Nucleic Acids Res. 61, 133 – 179
10. Birner, R., and Daum, G. (2003) Int. Rev. Cytol. 225, 273 – 323
11. Voeller, D. R. (2003) J. Lipid Res. 44, 441 – 449
12. Dowhan, W. (1997) Annu. Rev. Biochem. 66, 199 – 232
13. Dowhan, W., Milejkovskaya, E., and Bogdanov, M. (2004) Biochim. Biophys. Acta 1666, 19 – 39
14. Becker, G. W., and Lester, R. L. (1980) J. Bacteriol. 142, 747 – 754
15. Menon, A. K., and Stevens, V. L. (1992) J. Biol. Chem. 267, 15277 – 15280
16. Lester, R. L., and Dickson, R. C. (1993) Adv. Lipid Res. 26, 253 – 274
17. Fankhauser, C., Homann, S. W., Thomas-Oates, J. E., McConville, M. J., Desponds, C., Conzelmann, A., and Ferguson, M. A. (1993) J. Biol. Chem. 268, 26365 – 26374
18. Bishop, R. E., Gibbons, H. S., Guina, T., Trent, M. S., Miller, S. I., and Raetz, C. R. (2000) EMBO J. 19, 5071 – 5080
19. Bogdanov, M., and Dowhan, W. (1999) J. Biol. Chem. 274, 36827 – 36830
20. Bogdanov, M., Sun, J., Kaback, H. R., and Dowhan, W. (1996) J. Biol. Chem. 271, 11615 – 11618
21. Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Kominami, E., Ohsumi, M., Noda, T., and Ohsumi, Y. (2000) Nature 408, 488 – 492
22. Eston, J. H. (1994) Biochim. Biophys. Acta 1212, 26 – 42
23. Vallier, B. L., and Falchuk, K. H. (1993) Physiol. Rev. 73, 79 – 118
24. Schwabe, J. W., and Klug, A. (1994) Nat. Struct. Biol. 1, 345 – 349
25. Ellis, C. D., Wang, F., MacDiarmid, C. W., Clark, S., Lyons, T., and Eide, D. J. (2004) J. Cell Biol. 166, 325 – 335
26. Zhao, H., and Eide, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2454 – 2458
27. Zhao, H., and Eide, D. (1996) J. Biol. Chem. 271, 23203 – 23210
28. Waters, B. M., and Eide, D. J. (2002) J. Biol. Chem. 277, 33749 – 33757