Regulation of the PIS1-encoded Phosphatidylinositol Synthase in *Saccharomyces cerevisiae* by Zinc*

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In the yeast *Saccharomyces cerevisiae*, the mineral zinc is essential for growth and metabolism. Depletion of zinc from the growth medium of wild type cells results in changes in phospholipid metabolism including an increase in phosphatidylinositol content (Iwanyshyn, W.M., Han, G.-S., and Carman, G.M. (2004) J. Biol. Chem. 279, 21976-21983). We examined the effects of zinc depletion on the regulation of the PIS1-encoded phosphatidylinositol synthase, the enzyme that catalyzes the formation of phosphatidylinositol from CDP-diacylglycerol and inositol. Phosphatidylinositol synthase activity increased when zinc was depleted from the growth medium. Analysis of a zrt1Δ zrt2Δ mutant defective in plasma membrane zinc transport indicated that the cytoplasmic levels of zinc were responsible for the regulation of phosphatidylinositol synthase. PIS1 mRNA, its encoded protein Pis1p, and the β-galactosidase activity driven by the P*PIS1*-lacZ reporter gene were elevated in zinc-depleted cells. This indicated that the increase in phosphatidylinositol synthase activity was due to a transcriptional mechanism. The zinc-mediated induction of the P*PIS1*-lacZ reporter gene, Pis1p, and phosphatidylinositol synthase activity was lost in zap1Δ mutant cells. These data indicated that the regulation of PIS1 gene expression by zinc depletion was mediated by the zinc-regulated transcription factor Zap1p. Direct interaction between GST-Zap1p687-880 and a putative UASZRE in the PIS1 promoter was demonstrated by electrophoretic mobility shift assays. Mutations in the UASZRE in the PIS1 promoter abolished the GST-Zap1p687-880-DNA interaction *in vitro* and abolished the zinc-mediated regulation of the PIS1 gene *in vivo*. This work advances understanding of phospholipid synthesis regulation by zinc and the transcription control of the PIS1 gene.

Phosphatidylinositol (PI) is the third most abundant phospholipid in the cellular membranes of the yeast *Saccharomyces cerevisiae* (1-3), and it is essential for the growth and metabolism of this model eukaryote (4-6). In addition to being a major structural component of the membrane, PI serves as the precursor for sphingolipids (7, 8), the D-3, D-4, and D-5 phosphoinositides (3, 9-12), and glycosyl PI anchors (13, 14)(Fig. 1). Several of these PI-derived lipids and their metabolic products are prominent signaling molecules in *S. cerevisiae* and in higher eukaryotes that contribute to essential physiological functions (12, 15-20).

The enzyme responsible for the synthesis of PI in *S. cerevisiae* is the essential PIS1-encoded PI synthase (CDP-diacylglycerol:myo-inositol 3-phosphatidyltransferase, EC 2.7.8.11)(6, 21-23). This ER-associated (24) enzyme catalyzes the formation of PI and CMP from CDP-diacylglycerol and inositol (25) (Fig. 1). The regulation of PI synthase activity *in vivo* is largely governed by the availability of its substrates inositol and CDP-diacylglycerol (26-28). Cellular inositol levels are controlled by expression of the INO1 gene encoding inositol-3-phosphate synthase and by inositol...
supplementation (26-28). The levels of CDP-diacylglycerol are controlled through its utilization by the PI synthase enzyme itself and the competing activity of PS synthase (26-29)(Fig. 1). PS synthase catalyzes the committed step in the synthesis of PC via the CDP-diacylglycerol pathway (27) (Fig. 1). Indeed, the coordinate regulation of the PI synthase and PS synthase enzymes is part of an overall mechanism by which the synthesis of PI is coordinately regulated with the synthesis of PC (3, 27, 30-34).

Zinc is an essential nutrient required for the growth and metabolism of *S. cerevisiae* and of higher eukaryotes (35). It is a cofactor for hundreds of enzymes (e.g., alcohol dehydrogenase, carbonic anhydrase, proteases, RNA polymerases, superoxide dismutase) (35) and a structural constituent of many proteins (e.g., transcription factors, chaperones, lipid binding proteins)(36, 37). Zinc deficiency in rats is associated with oxidative damage to DNA, lipids, and proteins (38), and in humans, it is manifested by defects in appetite, cognitive function, embryonic development, epithelial integrity, and immune function (39). Despite its essential nature, zinc is toxic to cells when accumulated in excess amounts (35).

Recent studies have revealed that the synthesis of phospholipids in *S. cerevisiae* is influenced by zinc deficiency (40). In particular, PI synthase activity is elevated in zinc-depleted cells whereas several enzyme activities (e.g., PS synthase, PS decarboxylase, PE methyltransferase, and phospholipid methyltransferase) in the CDP-diacylglycerol pathway for PC synthesis are reduced in response to zinc depletion (40). The regulation of these activities by zinc availability contributes to alterations in the cellular levels of the major membrane phospholipids PI (elevated) and PE (reduced) (40). For the PS synthase enzyme, the reduction in activity in response to zinc depletion is controlled at the level of transcription through the UASINO element the *CHO1* promoter and by the transcription factors Ino2p, Ino4p, and Opi1p (40). In this work, we explored the mechanism by which PI synthase activity is regulated in response to zinc depletion. Our data indicated that this regulation occurred by a transcriptional mechanism that was mediated by the transcriptional activator 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 NEBlot kit were purchased from New England Biolabs, Inc. RNA size markers were purchased from Promega. The Yeastmaker<sup>™</sup> yeast transformation kit was obtained from Clontech. Plasmid DNA purification and DNA gel extraction kits were from Qiagen, Inc. The QuikChange<sup>™</sup> site-directed mutagenesis kit was from Stratagene. Oligonucleotides for PCRs and electrophoretic mobility shift assays were prepared by Genosys Biotechnology, Inc. ProbeQuant G-50 columns, polyvinylidene difluoride membranes, enhanced chemifluorescence Western blotting detection kit, and glutathione Sepharose<sup>™</sup> 4 fast flow were purchased from GE Healthcare. DNA markers for agarose gel electrophoresis, protein molecular mass standards for SDS-PAGE, Zeta Probe blotting membranes, protein assay reagents, electrophoretic reagents, immunochemical reagents, isopropyl-β-D-thiogalactoside, and acrylamide solutions were purchased from Bio-Rad. Ampicillin, aprotinin, benzamidine, bovine serum albumin, leupeptin, *O*-nitrophenyl β-D-galactopyranoside, pepstatin, phenylmethylsulfonyl fluoride, reduced glutathione, IGEPAL CA-630, and Triton X-100 were purchased from Sigma. Mouse monoclonal anti-HA antibodies (12CA5) and ImmunoPure goat anti-mouse IgG (H+L) antibodies were purchased from Roche and Pierce, respectively. Radiochemicals and scintillation counting supplies were purchased from PerkinElmer Life Sciences and National Diagnostics, respectively. Liqui-Nox detergent was from Alconox, Inc.

**Strains, Plasmids, and Growth Conditions—**The strains and plasmids used in this work are presented in Table I. Yeast cells were grown according to standard methods (41, 42) at 30 °C in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or in synthetic complete
medium containing 2% glucose. Appropriate nutrients were omitted from synthetic complete medium for the selection of cells bearing plasmids. Zinc-deplete medium was synthetic complete medium prepared with yeast nitrogen base lacking zinc sulfate. For zinc-depleted cultures, cells were first grown for 24 h in synthetic complete medium supplemented with 1.5 µM zinc sulfate. Standard synthetic growth medium contains 1.4 µM zinc sulfate. Saturated cultures were harvested, washed in deionized distilled water, diluted to 1 x 10^6 cells/ml in media containing 0 or 1.5 µM zinc sulfate, and grown for 24 h. Cultures were then diluted to 1 x 10^5 cells/ml and grown again in media containing 0 or 1.5 µM zinc sulfate. This growth routine with medium lacking zinc was used to deplete internal stores of zinc (43). Cells in liquid media were grown to the exponential phase (1 x 10^7 cells/ml), and cell numbers were determined spectrophotometrically at an absorbance of 600 nm. Plasmids were maintained and amplified in Escherichia coli strain DH5α 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 contained plasmids. Yeast and bacterial media were supplemented with 2% and 1.5% agar, respectively for growth on plates. Glassware were washed with Liqui-Nox, rinsed with 0.1 mM EDTA, and then rinsed several times with deionized distilled water to prevent zinc contamination.

**DNA Manipulations and Amplification of DNA by PCR**—Plasmid and genomic DNA preparation, restriction enzyme digestion, and DNA ligations were performed by standard methods (42). Conditions for the amplification of DNA by PCR were optimized as described previously (44). Transformation of yeast (45) and E. coli (42) were performed using standard protocols.

**Construction of Plasmids**—Plasmid pWMI1 contains a 2.2-kb DNA fragment for the PISI gene with sequences for an HA epitope tag inserted after the start codon. Genomic DNA prepared from strain W303-1A was used as a template to produce a 5' fragment of PISI HA (primers: 5'-AGCGTAGTCTGGGACGTATGGGTAC ATCTTGACTATCACACTTCTCCITTAT -3') and a 3' fragment of PISI HA (primers: 5'-TACCCTACAGCACCTCCAGACTACGCTA GTTCGAATTCACACAGAAAAAGGTTAC T-3' and 5'-CGTCTAGGTCAGTGGAGAGAGAAATCG CTTCCG-3'). The 5'- and 3'-fragments of PISI HA were digested with Xmal/AatII and AatII/XbaI, respectively, and inserted into the Xmal/XbaI sites of pRS416 to generate the plasmid pWMI1. The Stratagene QuikChange™ Site-Directed Mutagenesis Kit was utilized according to the manufacturer’s instructions to generate plasmids pPZM1-pPZM3. These plasmids were derivatives of pMA109 (P_{PISI} lacZ) and contained mutations in UAS\_ZRE, UAS\_ZRE^2, and UAS\_ZRE^3 of the PISI promoter. Plasmids pPZM1 (mutagenic primers: 5'- TTTTCTTCCTCTTCTCTCTCTTCTCTCTCTCTCTCTCT-3' and 5'- AAGGAGAGAGAGAGAGAGAGAGGCTTGGA ATGTGGAGGAAAAAGGAGAA AAAA-3'), pPZM2 (mutagenic primers: 5'- TTTTAGCCATGGACACTTCTCAATTCCTCATTGATTCGATGCTCAAAA-3' and 5'- TCAATGGCAGTTTATCACAACAAAGTTG GAATGGAGGAAAAAGGAGAAA AAAA-3'), and pPZM3 (mutagenic primers: 5'- ATATAAGTAAAACATAAAAACAATTCCA ATGGGTATGGTTTATTTGCCGTC-3' and 5'- GACGGCAAATAACCAATCACAAATTGG AA ATTTTTATATTTTTATATATATATATAT-3') were constructed by amplification of plasmid pMA109 by PCR. Plasmid pMA109 was eliminated from the mutant plasmid reactions by digestion with DpnI. The mutant plasmids were amplified in E. coli, and the purified plasmids were sequenced to confirm the mutations in the PISI promoter.

**RNA Isolation and Northern Blot Analysis**—Total RNA was isolated from cells (46, 47), resolved by agarose gel electrophoresis (48), and then transferred to Zeta Probe membranes by vacuum blotting. The PISI and CMD1 probes were labeled with [α-^32P]dTTP using the NEBlot random primer labeling kit, and unincorporated nucleotides were removed using ProbeQuant G-50 columns. Prehybridization, hybridization...
with the probes, and washes to remove nonspecific binding were carried out according to manufacturer’s instructions. Images of the radiolabeled mRNAs were acquired by phosphorimaging analysis.

**Anti-PI Synthase Antibodies and Immunoblotting**—The peptide sequence AALILADNDAKNANE (residues 201-215 at the C-terminal end of the deduced amino acid sequence of PIS1) was synthesized and used to raise antibodies in New Zealand White rabbits by standard procedures at Bio-Synthesis, Inc. The IgG fraction was isolated from the antiserum using protein A Sepharose™ CL-4B (49). SDS-PAGE (50) using 10% slab gels and the transfer of proteins to polyvinylidene difluoride membranes (51) were performed as described previously. The membrane was probed with 12.5 µg/ml of the purified anti-PI synthase IgG fraction. Mouse monoclonal anti-HA antibodies were used at a dilution of 1:1000. Goat anti-rabbit and anti-mouse IgG-alkaline phosphatase conjugates were used as secondary antibodies at a dilution of 1:5000. The PI synthase protein (Pis1p) was detected using the enhanced chemifluorescence Western blotting detection kit, and the signals were acquired by FluorImaging. The relative density of the signal was analyzed using ImageQuant software. Immunoblot signals were in the linear range of detectability.

**Preparation of Cell Extracts and Protein Determination**—Cell extracts were prepared as described previously (52). Cells were suspended in 50 mM Tris-maleate buffer (pH 7.0) 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. Cells were disrupted by homogenization with chilled glass beads (0.5 mm diameter) using a Biospec Products Mini-BeadBeater-8. Samples were homogenized for ten 1-min bursts followed by a 2-min cooling between bursts at 4 °C. The cell extract (supernatant) was obtained by centrifugation of the homogenate at 1,500 x g for 10 min. Protein concentration was determined by the method of Bradford (53) using bovine serum albumin as the standard.

**Enzyme Assays**—All assays were conducted in triplicate at 30 °C in a total volume of 0.1 ml. PI synthase activity was measured by following the incorporation of [2-3H]inositol (10,000 cpm/nmol) into PI as described previously (54). The assay mixture contained 50 mM Tris-HCl (pH 8.0), 2 mM MnCl2, 0.5 mM inositol, 0.2 mM CDP-diaclylglycerol, 2.4 mM Triton X-100, and enzyme protein. 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 (55). The assay mixture contained 100 mM sodium phosphate (pH 7.0), 3 mM O-nitrophenyl β-D-galactopyranoside, 1 mM MgCl2, 100 mM 2-mercaptoethanol, and enzyme protein. All assays were linear with time and protein concentration. The average standard deviation of all assays was ± 5%. A unit of PI synthase activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product/min whereas a unit of β-galactosidase activity was defined in µmol/min. Specific activity was defined as units/mg of protein.

**Expression and Purification of GST-Zap1p687-880 from E. coli**—The GST-Zap1p fusion protein was expressed in E. coli BL21(DE3)pLysS bearing plasmid pGEX-687. A 500-ml culture was grown to A600 ~ 0.8 at 28 °C, and the expression of GST-Zap1p687-880 was induced for 1 h with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. The culture was harvested, and the resulting pellet was resuspended in 20 ml of phosphate-buffered saline (10 mM Na2HPO4, 1.8 mM KH2PO4, 140 mM NaCl, 2.7 mM KCl, pH 7.3). Cells were disrupted with a French press at 20,000-pounds/square inch, and unbroken cells and cell debris were removed by centrifugation at 12,000 x g for 30 min at 4 °C. The supernatant (cell lysate) was mixed for 1 h with 1 ml of a 50% slurry of glutathione Sepharose with gentle shaking. The glutathione Sepharose resin was then packed in a 10-ml Poly-Prep disposable column and was washed with 25 ml of phosphate-buffered saline. Proteins bound to the column were eluted (0.5-ml fractions) with 50 mM Tris-HCl (pH 8.0) buffer containing 10 mM reduced glutathione. SDS-PAGE analysis
indicated that the 48-kDa GST- Zap1p fusion protein was purified to ~90% of homogeneity. The purified GST- Zap1p preparation was dialyzed against phosphate-buffered saline containing 10% glycerol and 2.5 mM dithiothreitol.

**Electrophoretic Mobility Shift Assays—**
Double-stranded oligonucleotides (Table II) were prepared by annealing 25 µM complementary single-stranded oligonucleotides in a total volume of 0.1 ml containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM EDTA. The reaction mixtures were incubated for 5 min at 100 °C in a heat block, and then for 2 h in the heat block that was turned-off. Annealed oligonucleotides were designed to contain a 5' overhanging end, and they were labeled by incorporating [α-32P]dTTP to the ends. Annealed oligonucleotides (100 pmol) were incubated with 5 units of Klenow fragment and [α-32P]dTTP (400-800 Ci/nmol) for 30 min at room temperature. Labeled oligonucleotides were purified from unincorporated nucleotides using ProbeQuant G-50 spin columns.

Formation of the protein-DNA complexes was allowed for 15 min at room temperature in a total volume of 10 µl containing 1 pmol of radiolabeled DNA probe (2.5 x 10^5 cpm/pmol), 10 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 50 mM KCl, 1 mM DTT, 0.025 mg/ml poly(dI-dC)·poly(dI-dC), 0.2 mg/ml bovine serum albumin, 0.04% IGEPLA CA-630, 10% glycerol, and the indicated concentrations of purified GST- Zap1p. The reaction mixtures were resolved on 6% polyacrylamide gels (1.5-mm thickness) in 0.5x 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.

**Analyses of Data**—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 the zrt1Δ zrt2Δ Mutations on the Expression of PI Synthase Activity in Response to Zinc Depletion**—Iwanyshyn et al. (40) identified PI synthase as an enzyme whose activity increased in wild type cells when zinc was depleted from the growth medium. To confirm that this regulation was governed by the intracellular levels of zinc, the expression of PI synthase activity was examined in a zrt1Δ zrt2Δ double mutant (56). This mutant lacks both the high-affinity (Zrt1p) and low-affinity (Zrt2p) plasma membrane zinc transporters that are primarily responsible for regulating the cytoplasmic levels of zinc in *S. cerevisiae* (56, 57). For this and subsequent experiments, the growth medium lacked inositol and choline supplementation to preclude the regulatory effects that these phospholipid precursors have on phospholipid synthesis (3, 27, 30, 31).

As described previously (40), depletion of zinc from the growth medium of wild type cells caused a 2-fold increase in the expression of PI synthase activity (Fig. 2). The level of PI synthase activity in the zrt1Δ zrt2Δ mutant grown in the presence of zinc was similar to that expressed in the wild type control cells that were depleted for zinc (Fig. 2). This result indicated that the intracellular levels of zinc were responsible for regulating the expression of PI synthase activity.

**Effect of Zinc Depletion on the Expression of PI Synthase Protein and PIS1 mRNA**—Antibodies were generated against a peptide sequence found at the C-terminal end of the PI synthase protein. These antibodies recognized a protein with a subunit molecular mass of 24 kDa, the predicted size of the *PIS1* gene product (6). To confirm the identity of this 24-kDa protein as the PI synthase protein, an immunoblot experiment was performed using a cell extract derived from wild type cells that overexpressed the *PIS1* gene on a high copy plasmid. Consistent with the overexpression of the *PIS1* gene, the amount of the 24-kDa protein that was recognized by the anti-PI synthase antibodies was elevated ~7-fold. As a further confirmation, an immunoblot experiment was performed using a cell extract from wild type cells that expressed the *PIS1 HA* gene on a single copy plasmid. The anti-PI synthase antibodies recognized both the native and HA-tagged versions of the PI synthase protein. HA-tagged PI synthase migrated with a molecular mass of 25 kDa because of the HA epitope. The identity of the HA-tagged PI synthase protein was...
confirmed by immunoblot analysis using anti-HA antibodies.

The expression of the PI synthase protein was analyzed by immunoblotting to examine the mechanism by which PI synthase activity was regulated in response to zinc depletion. Zinc depletion resulted in nearly a 2-fold increase in the amount of the PI synthase protein when compared with cells grown with zinc (Fig. 3A). This indicated that the increase in PI synthase activity was a result of an increase in enzyme level.

We next examined the level of \textit{PIS1} mRNA to determine if the increase in enzyme content was due to an increase in gene expression. \textit{CMD1} mRNA (encodes calmodulin) was measured in this analysis as a loading control because its expression level is not affected by zinc availability (58, 59). Northern blot analysis of total RNA isolated from exponential phase cells showed that the relative amount of \textit{PIS1} mRNA in zinc-depleted cells was almost 2-fold greater when compared with that found in cells grown with zinc (Fig. 3B). These results indicated that a transcriptional mechanism was responsible for the regulation of PI synthase in zinc-depleted cells.

\textit{Effect of Zinc Depletion on the Expression of} \textit{\beta}-\textit{galactosidase Activity in Cells Bearing the} \textit{P\textsc{pisi}-lacZ Reporter Gene}—The analysis of \textit{PIS1} expression was facilitated by the use of plasmid pMA109 that bears a \textit{P\textsc{pisi}-lacZ} reporter gene where the expression levels of \textit{\beta}-galactosidase activity are dependent on transcription driven by the \textit{PIS1} promoter (60). To further examine the effect of zinc depletion on the expression of the \textit{PIS1} gene, we measured \textit{\beta}-galactosidase activity from wild type cells bearing plasmid pMA109 that were grown with various concentrations of zinc. Reduction for zinc in the growth medium resulted in a dose-dependent increase in \textit{\beta}-galactosidase activity (Fig. 4). The activity found in cells grown in the absence of zinc was 3.5-fold greater than the activity in cells grown in the presence of 1.5 µM zinc (Fig. 4). Concentrations of zinc above 1.5 µM did not result in a further reduction in \textit{\beta}-galactosidase activity.

\textit{Effects of ino2\Delta, ino4\Delta, and opi1\Delta Mutations on the Regulation of PI synthase by Zinc Depletion}—The PI synthase enzyme is found at a branch point in phospholipid synthesis where it competes with another enzyme, PS synthase, for the common liponucleotide substrate CDP-diacylglycerol (27). Unlike \textit{PIS1}, the expression of the PS synthase gene (\textit{CHO1}) is repressed in wild type cells when zinc is depleted from the growth medium (40). The regulation of PS synthase expression by zinc depletion is mediated through a UAS\textsubscript{INO} element in the \textit{CHO1} promoter and by the positive transcription factors Ino2p and Ino4p, and the negative transcription factor Opi1p (40). Owing to the fact that the \textit{PIS1} promoter contains a UAS\textsubscript{INO} element (60) and that the synthesis of PI and PS is coordinately regulated in \textit{S. cerevisiae} (3, 27, 27, 30, 31), we questioned whether the regulation of PI synthase expression by zinc depletion was mediated by Ino2p, Ino4p, and Opi1p. To address this question, PI synthase activity was measured in \textit{ino2\Delta, ino4\Delta, and opi1\Delta} mutant cells that were grown in the presence and absence of zinc. In all three regulatory mutants, the PI synthase enzyme was elevated in response to zinc depletion similar to that observed in wild type cells (data not shown). These results indicated that the induction of PI synthase in zinc-depleted cells was not mediated by Ino2p, Ino4p, and Opi1p.

\textit{Effects of the zap1\Delta Mutation on the Regulation of PI synthase by Zinc Depletion}—Zap1p is a positive transcription factor that is maximally expressed in zinc-deplete cells and repressed in zinc-replete cells (61). Zap1p directly regulates UAS\textsubscript{ZRE}-containing genes (e.g., \textit{ZRT1, ZRT2, ZRT3, ZRC1, FET4, DPP1}) whose expression is induced by zinc depletion (43, 58, 62-64). Inspection of the \textit{PIS1} promoter revealed that it contains sequences that bear resemblance to the consensus UAS\textsubscript{ZRE} (see below). Accordingly, we questioned whether the regulation of PI synthase expression by zinc was dependent on Zap1p function. In the first set of experiments, the \textit{zap1\Delta} mutant bearing the \textit{P\textsc{pisi}-lacZ} reporter gene was grown in the presence and absence of zinc followed by the measurement of \textit{\beta}-galactosidase activity. In contrast to wild type cells, zinc depletion did not result in the induction of \textit{\beta}-galactosidase activity.
In a second set of experiments, PI synthase protein and activity levels were measured in cell extracts derived from zap1Δ mutant cells grown in the presence and absence of zinc. Unlike wild type cells, the depletion of zinc from the growth medium of the zap1Δ mutant did not result in elevated levels of PI synthase protein (Fig. 5B) and activity (Fig. 5C). These results indicated that the zinc-mediated regulation of PIS1 expression was dependent on the Zap1p transcription factor.

**Interactions of GST-Zap1p<sub>687-880</sub> with Putative UAS ZRE Sites in the PIS1 Promoter**

We sought evidence that Zap1p mediates the regulation of PIS1 expression in response to zinc depletion by direct interaction with the PIS1 promoter. The PIS1 promoter contains three putative UAS ZRE sites (UASZRE<sub>1</sub>, UASZRE<sub>2</sub>, and UASZRE<sub>3</sub>) with sequences that resemble the consensus UAS ZRE sequence for Zap1p binding (Fig. 6A). Electrophoretic mobility shift assays were performed with labeled oligonucleotides containing the putative UAS ZRE sites using recombinant GST-Zap1p<sub>687-880</sub> purified from E. coli. Zap1p<sub>687-880</sub> contains the UAS ZRE binding domain (amino acids 687-880) of Zap1p (65). Of the three probes, the oligonucleotide containing UAS ZRE<sub>3</sub> showed the strongest interaction with GST-Zap1p<sub>687-880</sub> (Fig. 6B). The interaction of GST-Zap1p<sub>687-880</sub> with UAS ZRE<sub>1</sub> was ~20-fold lower when compared with UAS ZRE<sub>3</sub> whereas an interaction with UAS ZRE<sub>2</sub> was hardly detectable (Fig. 6B). The interaction of GST-Zap1p<sub>687-880</sub> with UAS ZRE<sub>3</sub> was examined further using the same assay. The formation of the GST-Zap1p<sub>687-880</sub>-UAS ZRE<sub>3</sub> complex was dependent on the concentration of GST-Zap1p<sub>687-880</sub> (Fig. 7A). In addition, the unlabeled UAS ZRE<sub>3</sub> probe competed with the labeled probe for binding to GST-Zap1p<sub>687-880</sub> in a dose-dependent manner (Fig. 7B). Moreover, this interaction was abolished when the UAS ZRE<sub>3</sub> sequence was mutated (M1) to a nonconsensus sequence (Fig. 7C). When the UAS ZRE<sub>3</sub> sequence was mutated (M2) to the consensus UAS ZRE, the extent of interaction with GST-Zap1p<sub>687-880</sub> was 10-fold greater than the interaction with the wild type UAS ZRE<sub>3</sub> sequence (Fig. 7C).

**Effects of Mutations in the Putative UAS ZRE Elements in the PIS1 Promoter on the Zinc-mediated Regulation of PIS1 Expression**

The effects of mutations in UASZRE<sub>1</sub>, UASZRE<sub>2</sub>, and UASZRE<sub>3</sub> in the PIS1 promoter on the zinc-mediated regulation of PIS1 expression was examined. P<sub>PSI</sub>-lacZ reporter genes were constructed with mutations in each of the three putative UAS ZRE elements. For each element, the core sequences were changed to the nonconsensus sequence of 5'-CAATTCCAATT-3'. Cells bearing the wild type or mutant P<sub>PSI</sub>-lacZ reporter genes were grown in the presence and absence of zinc; cell extracts were prepared and assayed for β-galactosidase activity. The mutations in UAS ZRE<sub>3</sub> in the reporter plasmid pPZM3 abolished the induction of β-galactosidase activity that was observed in zinc-depleted cells bearing the wild type P<sub>PSI</sub>-lacZ reporter plasmid pMA109 (Fig. 8). Although the expression of the β-galactosidase activities found in cells bearing the reporter plasmids with mutations in UAS ZRE<sub>1</sub> (pPZM1) and UAS ZRE<sub>2</sub> (pPZM2) was somewhat attenuated, the PIS1 gene was still induced when cells were depleted for zinc (Fig. 8). These data indicated that the zinc-mediated regulation of PIS1 expression was primarily mediated by the UAS ZRE<sub>3</sub> sequence in its promoter.

**DISCUSSION**

The yeast S. cerevisiae has the ability to cope with a variety of stress conditions (e.g., nutrient deprivation) by regulating the expression of enzyme activities including those involved in phospholipid synthesis (4, 27, 40, 40, 43, 66, 67). In particular, the stress condition of zinc depletion results in an increase in PI content that is attributed to elevated expression of PI synthase activity (40). Analysis of the zrt1Δ zrt2Δ mutant defective in the major plasma membrane zinc transporters Zrt1p and Zrt2p indicated that a decrease in the intracellular levels of zinc was responsible for the induction of PI synthase activity. That PIS1 mRNA, its encoded protein Pis1p, and the β-galactosidase activity driven by the P<sub>PSI</sub>-lacZ reporter gene were elevated in zinc-depleted cells indicated that the increase in PI synthase activity was due to a transcriptional mechanism.
The zinc-mediated induction of the \( P_{\text{PISI}} \)-lacZ reporter gene, and PI synthase protein and activity was lost in \( \text{zap1} \Delta \) mutant cells. These data indicated that the regulation of \( P_{\text{PISI}} \) gene expression by zinc was mediated by the Zap1p transcription factor. Zap1p is a zinc-sensing and zinc-inducible regulatory protein that binds to a UAS\( _{\text{ZRE}} \) found in the promoter of regulated genes to drive their transcription (58, 61, 68-71). Zap1p plays a major role in regulating the intracellular levels of zinc in \( S. \text{cerevisiae} \) (61, 71). For example in zinc-depleted cells, Zap1p mediates increased expression and activity of the high-affinity (Zrt1p) and low-affinity (Zrt2p, Fet4p) zinc transporters in the plasma membrane and of the efflux zinc transporter Zrt3p in the vacuole membrane to elevate the cytoplasmic levels of zinc (56, 57, 62, 68, 71, 72).

The promoter of the \( P_{\text{PISI}} \) gene does not contain a consensus \( \text{UAS}_{\text{ZRE}} \). However, three putative \( \text{UAS}_{\text{ZRE}} \) sites were identified in the \( P_{\text{PISI}} \) promoter sequence by a motif search using the Vector NTI computer program. Electrophoretic mobility shift assays with DNA probes containing the putative \( \text{UAS}_{\text{ZRE}} \) sites and purified recombinant GST-Zap1p\(^{687-880}\) showed that \( \text{UAS}_{\text{ZRE}}^3 \) in the \( P_{\text{PISI}} \) promoter was required for GST-Zap1p\(^{687-880}\) binding \textit{in vitro}. Moreover, mutations in \( \text{UAS}_{\text{ZRE}}^3 \) to a nonconsensus sequence abolished the GST-Zap1p\(^{687-880}\)-DNA interactions \textit{in vitro} and abolished the induction of \( P_{\text{PISI}} \) gene expression (as reflected in \( \beta\)-galactosidase activity) in response to zinc depletion. A genome-wide cDNA microarray analysis of gene expression identified 46 direct Zap1p target genes that are induced by zinc depletion (58). The \( P_{\text{PISI}} \) gene was not identified in that microarray study (58). This might be attributed to the relatively modest level of \( P_{\text{PISI}} \) induction (~2-fold) when compared with the >10-fold inductions of other Zap1p target genes (e.g., ZRT1, DPP1) (43, 58).

The differences between the magnitudes of induction of the \( P_{\text{PISI}} \) gene and other Zap1p target genes correlated with the relative binding efficiencies of GST-Zap1p\(^{687-880}\) with the \( P_{\text{PISI}} \) promoter \( \text{UAS}_{\text{ZRE}}^3 \) sequence when compared with this sequence mutated to a consensus \( \text{UAS}_{\text{ZRE}} \) sequence. Notwithstanding, the 2-fold induction of the \( P_{\text{PISI}} \) gene in response to zinc depletion correlated with the ~2-fold increase in the PI content of yeast cells depleted for zinc (40). The steady state composition of PI in \( S. \text{cerevisiae} \) is tightly regulated (~2- to 3-fold changes) (2, 3, 27). In this regard, we found that the expression of PI synthase did not respond to zinc depletion when the \( P_{\text{PISI}} \) gene was overexpressed from a plasmid.

Inositol, the water-soluble substrate of the PI synthase enzyme reaction, plays a major role in the regulation of phospholipid synthesis and composition in \( S. \text{cerevisiae} \) (2-5, 27). The addition of inositol to the growth medium of wild type cells causes an increase in the level of PI and a decrease in the levels of PS, PE, and PC (28, 52). The decreased levels of PS, PE, and PC are primarily due to a repression mechanism that involves the positive transcription factors Ino2p and Ino4p, the negative transcription factor Opi1p, and a UASINO element found in the promoter of genes (i.e., \( \text{CHO1, PSD1, CHO2, and OPI3} \)) encoding the enzymes in the CDP-diacylglycerol pathway for PC synthesis (3, 27, 30-32) (Fig. 1). The coordinate repression of the CDP-diacylglycerol pathway enzymes by inositol requires the ongoing synthesis of PC (73, 74), and is enhanced by the inclusion of choline in the growth medium (3, 27, 30-32). The increased level of PI in response to inositol/choline supplementation is not due to increased expression of \( P_{\text{PISI}} \) mRNA (75) and the PI synthase enzyme (76). Transcription of the \( P_{\text{PISI}} \) gene is insensitive to inositol/choline, and it does not require the UASINO element in its promoter or the transcription factors Ino2p and Opi1p (60). The regulation of PI synthesis by inositol is due to a biochemical mechanism (28).

Given the low intracellular levels of inositol and the relatively high \( K_m \) value for inositol, the synthesis of PI by the PI synthase enzyme is regulated by the availability of inositol (28). Moreover, inositol is an inhibitor of the PS synthase enzyme, and this regulation also contributes to the decrease in the synthesis of PS and ultimately PE and PC (28). These observations raised the suggestion that PI synthase is a constitutively expressed enzyme (3, 30, 31). However, as shown here, the level of the PI synthase enzyme is regulated by zinc availability.

This is not the first study to show that the expression of the \( P_{\text{PISI}} \) gene is subject to
transcriptional regulation. Anderson and Lopes (60) have shown that expression of \textit{PIS1} is regulated in response to growth medium carbon source. When compared with glucose, glycerol represses \textit{PIS1} expression whereas galactose induces expression (60). The transcription factor Mcm1p mediates the glycerol-dependent repression of \textit{PIS1} gene expression whereas the transcription factor Sln1p mediates the galactose-mediated induction of gene expression (60). The expression of the \textit{PIS1} gene is also regulated by oxygen availability (77). Gene expression is induced when cells are grown under anaerobic conditions and repressed under aerobic conditions. Repression is dependent on transcription factor Rox1p and its binding site in the \textit{PIS1} promoter (77). Similar to that observed in cells deprived for zinc (40), a reduction in oxygen availability results in elevated levels of PI (77). The induction of \textit{PIS1} gene expression may represent one of the mechanisms by which cells cope with the stress conditions of zinc and oxygen deficiencies given that PI is a precursor to several lipid molecules (sphingolipids, phosphoinositides, and glycosyl PI anchors) that are essential to the growth and metabolism of this eukaryotic organism (3, 9-20).

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\textbf{REFERENCES}

1. Rattray, J. B., Schibeci, A., and Kidby, D. K. (1975) \textit{Bacteriol.Reviews} 39, 197-231
2. Henry, S. A. (1982) in \textit{The molecular biology of the yeast Saccharomyces: metabolism and gene expression} (Strathern, J. N., Jones, E. W., and Broach, J. R., eds) pp. 101-158, Cold Spring Harbor Laboratory, Cold Spring Harbor
3. Paltauf, F., Kohlwein, S. D., and Henry, S. A. (1992) in \textit{The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression} (Jones, E. W., Pringle, J. R., and Broach, J. R., eds) pp. 415-500, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
4. Becker, G. W. and Lester, R. L. (1977) \textit{J.Biol.Chem.} 252, 8684-8691
5. Henry, S. A., Atkinson, K. D., Kolat, A. J., and Culbertson, M. R. (1977) \textit{J.Bacteriol.} 130, 472-484
6. Nikawa, J., Kodaki, T., and Yamashita, S. (1987) \textit{J.Biol.Chem.} 262, 4876-4881
7. Dickson, R. C. (1998) \textit{Annu.Rev.Biochem.} 67, 27-48
8. Dickson, R. C. and Lester, R. L. (2002) \textit{Biochim.Biophys.Acta} 1583, 13-25
9. Fruman, D. A., Meyers, R. E., and Cantley, L. C. (1998) \textit{Annu.Rev.Biochem.} 67, 481-507
10. Balla, T. (1998) \textit{Biochim.Biophys.Acta} 1436, 69-85
11. Gehrmann, T. and Heilmayer, L. G., Jr. (1998) \textit{Eur.J.Biochem.} 253, 357-370
12. Odorizzi, G., Babst, M., and Emr, S. D. (2000) \textit{Trends Biochem.Sci.} 25, 229-235
13. Leidich, S. D., Drapp, D. A., and Orlean, P. (1994) \textit{J Biol.Chem.} 269, 10193-10196
14. Leidich, S. D. and Orlean, P. (1996) \textit{J Biol.Chem.} 271, 27829-27837
15. White, M. J., Lopes, J. M., and Henry, S. A. (1991) \textit{Adv.Microbiol.Physiol.} 32, 1-51
16. Lester, R. L. and Dickson, R. C. (1993) \textit{Adv.Lipid Res.} 26, 253-274
17. Downes, C. P. and Maephee, C. H. (1990) \textit{Eur.J.Biochem.} 193, 1-18
18. Divecha, N. and Irvine, R. F. (1995) \textit{Cell} 80, 269-278
19. Dove, S. K., Cooke, F. T., Douglas, M. R., Sayers, L. G., Parker, P. J., and Michell, R. H. (1997) \textit{Nature} 390, 187-192
20. Odom, A. R., Stahlberg, A., Wente, S. R., and York, J. D. (2000) \textit{Science} 287, 2026-2029
21. Nikawa, J. and Yamashita, S. (1984) \textit{Eur.J.Biochem.} 143, 251-256
22. Fischl, A. S. and Carman, G. M. (1983) \textit{J.Bacteriol.} 154, 304-311
23. Nikawa, J. and Yamashita, S. (1997) \textit{Biochim.Biophys.Acta Lipids Lipid Metab.} 1348, 173-178
24. Habeler, G., Natter, K., Thallinger, G. G., Crawford, M. E., Kohlwein, S. D., and Trajanoski, Z. (2002) \textit{Nucleic Acids Res.} 30, 80-83
25. Paulus, H. and Kennedy, E. P. (1960) \textit{J.Biol.Chem.} 235, 1303-1311
26. Carman, G. M. and Zeimetz, G. M. (1996) *J.Biol.Chem.* **271**, 13293-13296
27. Carman, G. M. and Henry, S. A. (1999) *Prog.Lipid Res.* **38**, 361-399
28. Kelley, M. J., Bailis, A. M., Henry, S. A., and Carman, G. M. (1988) *J.Biol.Chem.* **263**, 18078-18085
29. Loewen, C. J. R., Gaspar, M. L., Jesch, S. A., Delon, C., Ktistakis, N. T., Henry, S. A., and Levine, T. P. (2004) *Science* **304**, 1644-1647
30. Carman, G. M. and Henry, S. A. (1989) *Annu.Rev.Biochem.* **58**, 635-669
31. Greenberg, M. L. and Lopes, J. M. (1996) *Microbiol.Rev.* **60**, 1-20
32. Henry, S. A. and Patton-Vogt, J. L. (1998) *Prog.Nucleic Acid Res.* **61**, 133-179
33. Daum, G., Lees, N. D., Bard, M., and Dickson, R. (1998) *Yeast* **14**, 1471-1510
34. Kersting, M. C., Choi, H. S., and Carman, G. M. (2004) *J Biol.Chem.* **279**, 35353-35359
35. Vallee, B. L. and Falchuk, K. H. (1993) *Physiol Rev.* **73**, 79-118
36. Schwabe, J. W. and Klug, A. (1994) *Nat.Struct.Biol.* **1**, 345-349
37. Ellis, C. D., Wang, F., MacDiarmid, C. W., Clark, S., Lyons, T., and Eide, D. J. (2004) *J Cell Biol.* **166**, 325-335
38. Oteiza, P. L., Olin, K. L., Fraga, C. G., and Keen, C. L. (1996) *Proc.Soc.Exp.Biol.Med.* **213**, 85-91
39. Walsh, C. T., Sandstead, H. H., Prasad, A. S., Newberne, P. M., and Fraker, P. J. (1994) *Environ.Health Perspect.* **102**, 5-46
40. Iwanyshyn, W. M., Han, G. S., and Carman, G. M. (2004) *J.Biol.Chem.* **279**, 21976-21983
41. Rose, M. D., Winston, F., and Heiter, P. (1990) in *PCR Protocols. A Guide to Methods and Applications* (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds) pp. 3-12, Academic Press, Inc., San Diego
42. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
43. Han, G.-S., Johnston, C. N., Chen, X., Athenstaedt, K., Daum, G., and Carman, G. M. (2001) *J.Biol.Chem.* **276**, 10126-10133
44. Innis, M. A. and Gelfand, D. H. (1990) in *PCR Protocols. A Guide to Methods and Applications* (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds) pp. 3-12, Academic Press, Inc., San Diego
45. Haid, A. and Suissa, M. (1983) *Methods Enzymol.* **96**, 192-205
46. Klig, L. S., Homann, M. J., Carman, G. M., and Henry, S. A. (1985) *J.Bacteriol.* **162**, 1135-1141
47. Bradford, M. M. (1976) *Anal.Biochem.* **72**, 248-254
48. Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685
49. Haid, A. and Suissa, M. (1983) *Methods Enzymol.* **96**, 192-205
50. Klig, L. S., Homann, M. J., Carman, G. M., and Henry, S. A. (1985) *J.Bacteriol.* **162**, 1135-1141
51. Bradford, M. M. (1970) *Anal.Biochem.* **72**, 248-254
52. Craven, G. R., Steers, E., Jr., and Anfinsen, C. B. (1965) *J.Biol.Chem.* **240**, 2468-2477
53. Zhao, H. and Eide, D. (1996) *J.Biol.Chem.* **271**, 23203-23210
54. Zhao, H. and Eide, D. (1996) *Proc.Natl.Acad.Sci.U.S.A* **93**, 2454-2458
55. Bird, A. J., Blankman, E., Stillman, D. J., Eide, D. J., and Winge, D. R. (2004) *Proc.Natl.Acad.Sci.U.S.A* **97**, 7957-7962
56. Bird, A. J., Blankman, E., Stillman, D. J., Eide, D. J., and Winge, D. R. (2004) *EMBO J.* **23**, 1123-1132
57. Anderson, M. S. and Lopes, J. M. (1996) *J.Biol.Chem.* **271**, 26596-26601
58. Zhao, H. and Eide, D. J. (1997) *Mol.Cell Biol.* **17**, 5044-5052
59. Zhao, H. and Eide, D. J. (2002) *J.Biol.Chem.* **277**, 33749-33757
60. MacDiarmid, C. W., Milanick, M. A., and Eide, D. J. (2003) *J.Biol.Chem.* **278**, 15065-15072
61. Miyabe, S., Izawa, S., and Inoue, Y. (2000) *Biochem.Biophys.Res.Commun.* **276**, 879-884
62. Bird, A., Evans-Galea, M. V., Blankman, E., Zhao, H., Luo, H., Winge, D. R., and Eide, D. J. (2000) *J.Biol.Chem.* **275**, 16160-16166
66. Griac, P. and Henry, S. A. (1999) *Nucleic Acids Res.* **27**, 2043-2050
67. Homann, M. J., Poole, M. A., Gaynor, P. M., Ho, C.-T., and Carman, G. M. (1987) *J.Bacteriol.* **169**, 533-539
68. Zhao, H., Butler, E., Rodgers, J., Spizzo, T., Dueterhoeft, S., and Eide, D. (1998) *J.Biol.Chem.* **273**, 28713-28720
69. Bird, A. J., Zhao, H., Luo, H., Jensen, L. T., Srinivasan, C., Evans-Galea, M., Winge, D. R., and Eide, D. J. (2000) *EMBO J.* **19**, 3704-3713
70. Bird, A. J., McCall, K., Kramer, M., Blankman, E., Winge, D. R., and Eide, D. J. (2003) *EMBO J.* **22**, 5137-5146
71. Eide, D. J. (2003) *J.Nutr.* **133**, 1532S-1535S
72. MacDiarmid, C. W., Gaither, L. A., and Eide, D. (2000) *EMBO J.* **19**, 2845-2855
73. Gaynor, P. M., Gill, T., Toutenhoofd, S., Summers, E. F., McGraw, P., Homann, M. J., Henry, S. A., and Carman, G. M. (1991) *Biochim.Biophys.Acta* **1090**, 326-332
74. Morash, S. C., McMaster, C. R., Hjelmstad, R. H., and Bell, R. M. (1994) *J.Biol.Chem.* **269**, 28769-28776
75. Kodaki, T., Hosaka, K., Nikawa, J., and Yamashita, S. (1991) *J.Biochem.* **109**, 276-287
76. Fischl, A. S., Homann, M. J., Poole, M. A., and Carman, G. M. (1986) *J.Biol.Chem.* **261**, 3178-3183
77. Gardocki, M. E. and Lopes, J. M. (2003) *J Biol.Chem.* **278**, 38646-38652
78. Thomas, B. and Rothstein, R. (1989) *Cell* **56**, 619-630
79. Sikorski, R. S. and Hieter, P. (1989) *Genetics* **122**, 19-27
FOOTNOTES

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1The abbreviations used are: PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; UASZRE, zinc-responsive element; UASINO, inositol-responsive element.
| Strain or plasmid | Relevant characteristics | Reference |
|-------------------|--------------------------|-----------|
| **S. cerevisiae** |                          |           |
| W303-1A           | MAT\(a\) ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 | (78)      |
| DY1457            | MAT\(a\) ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52 | (61)      |
| ZHY6              | MAT\(a\) ade6 can1-100oc his3 leu2 ura3 zap1\(\Delta::\)TRP1 | (61)      |
| ZHY3              | MAT\(a\) ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52 zrt1\(\Delta::\)LEU2 zrt2\(\Delta::\)HIS3 | (56)      |
| SH303             | MAT\(a\) his3\(\Delta200\) leu2\(\Delta1\) trp1\(\Delta63\) ura3-52 ino2\(\Delta::\)TRP1 | S.A. Henry |
| SH307             | MAT\(a\) his3\(\Delta200\) leu2\(\Delta1\) trp1\(\Delta63\) ura3-52 ino4\(\Delta::\)LEU2 | S.A. Henry |
| SH304             | MAT\(a\) his3\(\Delta200\) leu2\(\Delta1\) trp1\(\Delta63\) ura3-52 opi1\(\Delta::\)LEU2 | S.A. Henry |
| **E. coli**       |                          |           |
| DH5\(\alpha\)     | F\(^+\) \(\phi80\)lacZ\(\Delta M15\) \((\text{lac}Z\text{YA-argF})\)U169 deoR, rec\(A1\) end\(A1\) \(hdR17\)(\(r\(_\text{y}^{-}\) \(m\(_\text{i}^{-}\)\)) phoA sup\(E44\) \(\lambda\) thi-1 gyr\(A96\) rel\(A1\) | (42)      |
| **Plasmids**      |                          |           |
| pWMI1             | HA-tagged PISI gene ligated into the XmaI/XbaI sites of pRS416 | This study |
| pRS416            | Single-copy E. coli/yeast shuttle vector containing URA3 | (79)      |
| pPI514            | PISI gene on a multicopy plasmid with LEU2 | (21)      |
| pGEX-687          | E. coli expression plasmid for recombinant GST-Zap1\(p_{687-880}\) | (65)      |
| pMA109            | P\(_{PISI}\)-lacZ reporter plasmid containing the PISI promoter with URA3 | (60)      |
| pPZM1             | Derivative of pMA109 with mutations in UAS\(_{ZRE}^1\) | This study |
| pPZM2             | Derivative of pMA109 with mutations in UAS\(_{ZRE}^2\) | This study |
| pPZM3             | Derivative of pMA109 with mutations in UAS\(_{ZRE}^3\) | This study |
| Element | Annealed oligonucleotides<sup>a</sup> |
|---------|-------------------------------------|
| PIS1 UAS<sub>ZRE</sub><sup>1</sup> | 5’-TTCCCTAAACCTTTTCAGAGCTCtct-3’<br>3’-agaGGATTTGGAAAAGTCTCAGAGA-5’ |
| PIS1 UAS<sub>ZRE</sub><sup>2</sup> | 5’-GACACTTCTATCTTGAAGTGGTGTGata-3’<br>3’-ctgTGAAGATAGATCTTCCAACTAT-5’ |
| PIS1 UAS<sub>ZRE</sub><sup>3</sup> | 5’-CATAAAAACATGAGAGGTGTTGATggt-3’<br>3’-gtaTTTTTGTACTCTCCACCATAACCA-5’ |
| PIS1 UAS<sub>ZRE</sub><sup>3</sup> (M1) | 5’-CATAAAAACATGCCATTTGTTGATggt-3’<br>3’-gtaTTTTTTGTTAAGGTTAACCATACCA-5’ |
| PIS1 UAS<sub>ZRE</sub><sup>3</sup> (M2) | 5’-CATAAAAACATGCCATTGTTGATggt-3’<br>3’-gtaTTTTTTGGAACCTCCACCATAACCA-5’ |

<sup>a</sup>Underlined sequences are putative UAS<sub>ZRE</sub> sites. The mutations (M1 and M2) in UAS<sub>ZRE</sub><sup>3</sup> are shown in bold letters. The small letters indicate the nucleotides filled with the Klenow fragment.
FIG. 1. **Pathways for the synthesis of PI and PC in S. cerevisiae.** The pathways shown for the synthesis of PI and PC (CDP-diacylglycerol pathway) include the relevant steps discussed throughout this paper. The genes encoding enzymes responsible for the reactions in the pathways are indicated in the figure. A more detailed description of phospholipid synthesis that includes the Kennedy pathway for PC synthesis can be found in Refs 3 and 30. PA, phosphatidate; CDP-DAG, CDP-diacylglycerol; GPI, glycosyl phosphatidylinositol.

FIG. 2. **Effect of the zrt1Δ zrt2Δ mutations on the expression of PI synthesize activity in response to zinc depletion.** Wild type and zrt1Δ zrt2Δ mutant cells were grown in the presence (1.5 µM) and absence of zinc as indicated. Cell extracts were prepared and used for the assay of PI synthesize activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D.

FIG. 3. **Effect of zinc depletion on the expression of PI synthesize protein and PIS1 mRNA.** Wild type cells were grown in the presence (1.5 µM) and absence of zinc. Panel A, cell extracts were prepared and 50-µg samples were used for immunoblot analysis using anti-PI synthesize antibodies (12.5 µg/ml). A portion of the immunoblot is shown, and the position of the PI synthesize (Pis1p) protein is indicated. The signals of the PI synthesize protein from cells grown with and without zinc were quantified using ImageQuant software. The amount of PI synthesize protein found in cells grown with zinc was arbitrarily set at 1. The data shown is representative of two independent experiments. Panel B, total RNA was extracted and 25-µg samples were used for Northern blot analysis to determine the abundance of PIS1 mRNA. Portions of Northern blots are shown and the positions of PIS1 and CMD1 (loading control) mRNAs are indicated. The relative amounts of PIS1 and CMD1 mRNAs from cells grown with and without zinc were determined by ImageQuant analysis of the data. The relative amount of PIS1 to CMD1 mRNA in cells grown with zinc was arbitrarily set at 1. The data shown are representative of two independent experiments.

FIG. 4. **Dose-dependent induction of β-galactosidase activity in cells bearing the P_{PIS1}-lacZ reporter gene in response to zinc depletion.** Wild type cells bearing the P_{PIS1}-lacZ reporter plasmid pMA109 were grown in the absence and presence of the indicated concentrations of zinc sulfate. Cell extracts were prepared and used for the assay of β-galactosidase activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D.

FIG. 5. **Effect of the zap1Δ mutation on the regulation of PI synthesize by zinc depletion.** Wild type and zap1Δ mutant cells were grown in the presence (1.5 µM) and absence of zinc. Panel A, cell extracts were prepared from cells bearing the P_{PIS1}-lacZ reporter plasmid pMA109 and used for the assay of β-galactosidase activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D. Panel B, 50-µg samples of cell extracts were used for immunoblot analysis using anti-PI synthesize antibodies (12.5 µg/ml). The signals of the PI synthesize protein from wild type and zap1Δ mutant cells grown with and without zinc were quantified using ImageQuant software. The amount of PI synthesize protein found in wild type cells grown with zinc was arbitrarily set at 1. The data shown are representative of two independent experiments. Panel C, cell extracts were prepared and assayed for PI synthesize activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D.

FIG. 6. **Interactions of GST-Zap1p with putative UASZRE sites in the PIS1 promoter.** Panel A, the locations, and sequences of the putative UASZRE sites in the PIS1 promoter are shown in the figure.
T, TATA box. Panel B, samples (1 pmol) of double-stranded synthetic oligonucleotides (2.5 x 10^5 cpm/pmol) with sequences for UAS_{ZRE}^1 (1), UAS_{ZRE}^2 (2), and UAS_{ZRE}^3 (3) in the PIS1 promoter were incubated with 0.6 µg of recombinant GST-Zap1p_{687-880} purified from E. coli. Interaction of GST-Zap1p_{687-880} 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.

FIG. 7. Interactions of GST-Zap1p_{687-880} with UAS_{ZRE}^3. Samples (1 pmol) of radiolabeled double-stranded synthetic oligonucleotide (2.5 x 10^5 cpm/pmol) with the sequence for UAS_{ZRE}^3 in the PIS1 promoter were incubated with recombinant GST-Zap1p_{687-880}. Panel A, the experiment was performed with 0, 0.15, 0.3, and 0.6 µg of recombinant GST-Zap1p_{687-880}. Panel B, the experiment was performed with 0.6 µg of recombinant GST-Zap1p_{687-880} and 0, 25, 50, and 100 pmol of unlabeled oligonucleotide with the sequence for UAS_{ZRE}^3. Panel C, the experiment was performed with 0.6 µg of recombinant GST-Zap1p_{687-880} and sequences for wild type and mutated forms of UAS_{ZRE}^3. The wild type (WT) UAS_{ZRE}^3 sequence was mutated from 5'-ACATGAGAGGT-3' to the nonconsensus sequence 5'-CAATTCCAATT-3' (M1) and to a consensus sequence 5'-ACCTTGAAGGT-3' (M2). Interaction of GST-Zap1p_{687-880} 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.

FIG. 8. Effects of mutations in UAS_{ZRE}^1, UAS_{ZRE}^2, and UAS_{ZRE}^3 in the PIS1 promoter on the zinc-mediated regulation of β-galactosidase activity in cells bearing the P_PIS1-lacZ reporter gene. Wild type cells bearing the indicated P_PIS1-lacZ reporter plasmids were grown in the presence (1.5 µM) and absence of zinc. The UAS_{ZRE}^1, UAS_{ZRE}^2, and UAS_{ZRE}^3 sequences in the PIS1 promoter of plasmid pMA109 were mutated to the nonconsensus sequence 5'-CAATTCCAATT-3' in plasmids pPZM1, pPZM2, and pPZM3, respectively. Cell extracts were prepared and used for the assay of β-galactosidase activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments± S.D.
Fig. 1

Glucose-6-phosphate → Inositol-3-phosphate

\[ \text{INO1} \]

Inositol-3-phosphate → Inositol CMP

\[ \text{INM1} \]

Inositol CMP → CDP-DAG

\[ \text{PIS1} \]

CDP-DAG → Serine CMP

\[ \text{CHO1} \]

Serine CMP → PS

\[ \text{PSD1, PSD2} \]

PS → CO₂

PE → CHO2

OPI3 → PC
Fig. 2

![Graph showing PI Synthase activity in WT and zrt1Δ zrt2Δ strains with and without Zn](#)
Fig. 3

(A) Zn + vs. Zn - effect on Pis1p levels.

(B) Zn + vs. Zn - effect on PIS1 and CMD1 expression.
Fig. 4

A bar graph shows the activity of β-galactosidase, U/mg, in response to varying concentrations of ZnSO₄ (µM). The x-axis represents the concentration of ZnSO₄ (µM) with values of 1.5, 0.5, 0.1, and 0.0. The y-axis represents the activity of β-galactosidase, with values ranging from 0.0 to 0.4. The graph indicates an increase in β-galactosidase activity as the concentration of ZnSO₄ decreases.
Fig. 5

A. 

B. 

C. 

\(\beta\)-galactosidase, U/mg

Pis1p, Relative Amount

PI Synthase, U/mg
A

-800  -600  -400  -200  T

1  2  3

UAS_{ZRE}^1: ACCTTTTCAGA
UAS_{ZRE}^2: ATCTTAGAAGT
UAS_{ZRE}^3: ACATGAGAGGT
Consensus: ACCTTGAAGGT

B

UAS_{ZRE}

1  2  3

UAS_{ZRE} complex

GST-Zap1p^{687-880}
Fig. 7

Panel A: GST-Zap1p<sup>687-880</sup>

Panel B: UAS<sub>ZRE</sub><sup>3</sup>

Panel C: GST-Zap1p<sup>687-880</sup>-UAS<sub>ZRE</sub><sup>3</sup> complex

WT M1 M2

UAS<sub>ZRE</sub><sup>3</sup> probe
Fig. 8

The figure shows a bar chart depicting the activity of β-galactosidase in relation to different reporter plasmids and zinc concentrations.

- **pMA109**
  - + Zn: Activity level
  - - Zn: Activity level

- **pPZM1**
  - + Zn: Activity level
  - - Zn: Activity level

- **pPZM2**
  - + Zn: Activity level
  - - Zn: Activity level

- **pPZM3**
  - + Zn: Activity level
  - - Zn: Activity level

The y-axis represents the activity of β-galactosidase in units per mg (U/mg), ranging from 0.0 to 0.3 units. The x-axis lists the reporter plasmids.
Regulation of the PIS1-encoded phosphatidylinositol synthase in Saccharomyces cerevisiae by zinc

Seung-Hee Han, Gil-Soo Han, Wendy M. Iwayshyn and George M. Carman

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