Regulation of Phospholipid Synthesis in *Saccharomyces cerevisiae* by Zinc*

Wendy M. Iwanyshyn, Gil-Soo Han, and George M. Carman‡

From the Department of Food Science, Rutgers University, New Brunswick, New Jersey 08901

Zinc is an essential nutrient required for the growth and metabolism of eukaryotic cells. In this work, we examined the effects of zinc depletion on the regulation of phospholipid synthesis in the yeast *Saccharomyces cerevisiae*. Zinc depletion resulted in a decrease in the activity levels of the CDP-diacetylgllycerol pathway enzymes phosphatidylserine synthase, phosphatidylserine decarboxylase, phosphatidylethanolamine methyltransferase, and phospholipid methyltransferase. In contrast, the activity of phosphatidylinositol synthase was elevated in response to zinc depletion. The level of Aut7p, a marker for the induction of autophagy, was also elevated in zinc-depleted cells. For the *CHO1*-encoded phosphatidylserine synthase, the reduction in activity in response to zinc depletion was controlled at the level of transcription. This regulation was mediated through the UAS

\[ \text{IN0} \]

and by the transcription factors Ino2p, Ino4p, and Opi1p that are responsible for the inositol-mediated regulation of UAS

\[ \text{IN0} \]

-containing genes involved in phospholipid synthesis. Analysis of the cellular composition of the major membrane phospholipids showed that zinc depletion resulted in a 66\% decrease in phosphatidylethanolamine and a 29\% decrease in phosphatidylserine. A \[ \text{Zrt1}\Delta \text{Zrt22} \]

mutant (defective in the plasma membrane zinc transporters \[ \text{Zrt1p} \] and \[ \text{Zrt2p} \]) grown in the presence of zinc exhibited a phospholipid composition similar to that of wild type cells depleted for zinc. These results indicated that a decrease in the cytoplasmic levels of zinc was responsible for the alterations in phospholipid composition.

Phospholipids are amphipathic molecules that are major structural components of cellular membranes (1). In addition, phospholipids provide precursors for the synthesis of macromolecules (2–6), serve as molecular chaperons (7, 8), serve in structural components of cellular membranes (1). In addition, zinc deficiency in rats is associated with oxidative damage to DNA, lipids, and proteins (47), as well as causing a decrease in the overall phospholipid content (48–50). Although zinc is an essential nutrient, it can be toxic when accumulated in excess amounts (44). The cytoplasmic levels of zinc in yeast are controlled by a variety of mechanisms, including cellular influx (51), efflux (52, 53), and chelation by metallothioneins (54). The cytoplasmic levels of zinc in yeast are controlled by a variety of mechanisms, including cellular influx (51), efflux (52, 53), and chelation by metallothioneins (54). The cytoplasmic levels of zinc are largely controlled by high affinity and low affinity zinc transporters (Zrt1p and Zrt2p) in the plasma membrane (55, 56). Zrt1p and Zrt2p are induced when the extraacellular concentration of zinc is low (55, 56). Cytoplasmic levels of zinc are controlled further by the vacuole membrane efflux transporter Zrt3p. Expression of the vacuole membrane-associated

\[ \text{DPP1} \]

-encoded DGPP phosphatase (43, 57), a novel enzyme in yeast phospholipid metabolism (58), is also induced by zinc depletion (43). The induction of DGPP phosphatase expression in

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†To whom correspondence should be addressed: Dept. of Food Science, Rutgers University, 65 Dudley Rd., New Brunswick, NJ 08901. Tel.: 732-932-9611 (ext. 217); E-mail: carman@aesop.rutgers.edu.

‡The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CDP-DAG, CDP-diacetylglcerol; PA, phosphatidate; DGPP, diacylglycerol pyrophosphate.

§The DGPP phosphatase enzyme catalyzes the dephosphorylation of the β-phosphate from DGPP to form PA and then removes the phosphate from PA to form diacylglycerol (58).
zinc-depleted cells correlates with diminished levels of the minor vacuolar membrane phospholipids DGPP and PA (59). In addition to the changes in DGPP and PA, zinc depletion results in a reduction in the level of PE and an increase in the level of PI in the vacuole membrane (59). Analysis of dpp1Δ mutant cells depleted for zinc indicates that the alterations in the major vacuolar membrane phospholipids are not dependent on the regulation of DPP1 expression (60). In this work, we showed that zinc depletion resulted in a decrease in the activities of CDP-DAG pathway enzymes and an increase in the activity of PI synthase. The level of Aut7p, a marker for the induction of autophagy, was also measured. For zinc-depleted cultures, cells were first grown for 24 h in medium containing 0 or 1.4 mM zinc and then rinsed several times with deionized distilled water. For growth on plates, yeast and bacterial media were supplemented with 2 and 1.5% agar, respectively. To prevent zinc contamination, glassware was washed with Liqui-Nox, rinsed with 0.1 mM EDTA, and then rinsed several times with deionized distilled water.

**DNA Manipulations, RNA Isolation, and Northern Blot Analysis**—Plasmid and genomic DNA were prepared according to standard protocols (62). Transformations of yeast (63) and E. coli (62) were performed as described previously. Total RNA was isolated from cells as described previously (64, 65). The RNA was resolved by agarose gel electrophoresis (66) and then transferred to Zeta Probe membranes by vacuum blotting. The CHO1 (20) probe was labeled with [32P]dCTP 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 the manufacturer’s instructions. Radioactive images were acquired by phosphorimaging.

**Preparation of Cell Extracts and Protein Determination**—Cell extracts were prepared as described previously (67, 68). 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 μM aprotinin, 5 μM leupeptin, and 5 μM pepstatin. Cells were disrupted by homogenization with chilled glass beads (0.5 mm diameter) using a BIspec Products Mini-Bead Beater-8. Samples were homogenized for 10 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 × g for 10 min. Protein concentration was determined by the method of Bradford (69) 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. CDP-DAG synthase activity was measured with 50 mM Tris maleate buffer (pH 6.5), 20 mM MgCl₂, 0.5 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 μM aprotinin, 5 μM leupeptin, and 5 μM pepstatin. Cells were disrupted by homogenization with chilled glass beads (0.5 mm diameter) using a BIspec Products Mini-Bead Beater-8. Samples were homogenized for 10 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 × g for 10 min. Protein concentration was determined by the method of Bradford (69) using bovine serum albumin as the standard.
**Fig. 1. Effect of zinc depletion on the expression of phospholipid biosynthetic enzyme activities.** The pathways shown for the synthesis of phospholipids include the CDP-DAG and Kennedy pathways and the relevant steps discussed throughout this paper. A more detailed description of the pathway and phospholipid structures can be found in Ref. 12. Cell extracts were prepared from wild type (strain DY1457) cells grown in the presence and absence of zinc and used for the assay of CDP-DAG synthase (CDS), PS synthase (PSS), PS decarboxylase (PSD), PE methyltransferase (PEMT), phospholipid methyltransferase (PLMT), and PI synthase (PIS) activities as described under “Experimental Procedures.” The relative quantities of 32P-labeled phospholipids were analyzed using ImageQuant software.

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

**RESULTS**

**Effect of Zinc Depletion on the Expression of Phospholipid Biosynthetic Enzyme Activities**—The effects of zinc depletion on the expression of several phospholipid biosynthetic enzyme activities were examined in wild type cells. In these experiments, cells were grown without inositol and choline supplementation to preclude the regulatory effects that these phospholipid precursors have on phospholipid synthesis (11–13, 24).

**Regulation of Phospholipid Synthesis by Zinc**

*Effect of zinc depletion on the expression of phospholipid biosynthetic enzyme activities.* The pathways shown for the synthesis of phospholipids include the CDP-DAG and Kennedy pathways and the relevant steps discussed throughout this paper. A more detailed description of the pathway and phospholipid structures can be found in Ref. 12. Cell extracts were prepared from wild type (strain DY1457) cells grown in the presence and absence of zinc and used for the assay of CDP-DAG synthase (CDS), PS synthase (PSS), PS decarboxylase (PSD), PE methyltransferase (PEMT), phospholipid methyltransferase (PLMT), and PI synthase (PIS) activities as described under “Experimental Procedures.” The specific activities (nmol/min/mg) of these enzymes from cells grown with zinc were 0.62 ± 0.04, 0.82 ± 0.05, 0.02 ± 0.001, 0.70 ± 0.003, 0.53 ± 0.002, and 7.05 ± 0.77, respectively. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D.

**Effects of Zinc Depletion on the Expression of PS Synthase Protein and CHO1 mRNA**—The expression of the PS synthase enzyme was examined to gain insight into the mechanism by which the phospholipid biosynthetic enzyme activities were regulated by zinc depletion. PS synthase was chosen as a representative enzyme because it catalyzes the committed step in the CDP-DAG pathway (Fig. 1). The activity of CDP-DAG synthase, which is responsible for the formation of CDP-DAG (92), was not significantly affected by zinc depletion (Fig. 1). On the other hand, the activity of PI synthase (93), which competes with PS synthase for the substrate CDP-DAG (94), was elevated by 2-fold in zinc-depleted cells (Fig. 1).

**Effect of Zinc Depletion on the Expression of PS Synthase Protein and CHO1 mRNA**—The expression of the PS synthase enzyme was examined to gain insight into the mechanism by which the phospholipid biosynthetic enzyme activities were regulated by zinc depletion. PS synthase was chosen as a representative enzyme because it catalyzes the committed step in the CDP-DAG pathway (Fig. 1), and its gene expression is coordinately regulated with other genes in the pathway (11–13, 24–26). The levels of the PS synthase protein (Cho1p) were compared by immunoblot analysis of cell extracts derived from exponential phase cells that were grown in the presence and absence of zinc. Zinc depletion resulted in a 46% decrease in the amount of the PS synthase protein when compared with cells grown with zinc (Fig. 2). This indicated that the decrease in PS synthase activity was a result of a decrease in enzyme level. We examined the level of CHO1 mRNA to determine whether the decrease in enzyme content was due to a decrease in gene expression. Northern blot analysis of total RNA isolated from exponential phase cells showed that the relative amount of CHO1 mRNA in zinc-depleted cells was 60% lower than that...
found in cells grown with zinc (Fig. 2). These results indicated that a transcriptional mechanism was responsible for the regulation of PS synthase in zinc-depleted cells.

Effect of the zap1Δ Mutation on the Regulation of PS Synthase by Zinc Depletion—The expression of many *S. cerevisiae* genes is both positively and negatively regulated by zinc depletion (95). Zap1p is a transcription factor (96) that directly regulates UAS ZRE-containing genes (*e.g.* ZRT1, ZRT2, ZRT3, ZRC1, ETF4, and DPP1), whose expression is induced by zinc depletion (43, 95, 97–99). Although the *CHO1* gene does not contain the UAS ZRE in its promoter and its expression was repressed by zinc depletion, we questioned whether this regulation was indirectly mediated by Zap1p. To address this question, PS synthase activity was measured in *zap1Δ* mutant cells that were grown in the presence and absence of zinc. Although PS synthase activity was slightly higher (25%) in the *zap1Δ* mutant when compared with the wild type control, the enzyme was repressed by zinc depletion as in wild type cells. The level of PS synthase activity in the mutant was reduced (50%) in response to zinc depletion (Fig. 3). These results indicated that the repression of PS synthase in zinc-depleted cells was not mediated by Zap1p.

Effects of the UASINO Element and the Transcription Factors Ino2p, Ino4p, and Opi1p on the Regulation of PS Synthase Expression by Zinc Depletion—A cis-acting element, UASINO, in the *CHO1* promoter is required for maximum expression of the *CHO1* gene in wild type cells grown in the absence of inositol (11, 24, 100). It contains a consensus-binding site (5′- CANNNTG-3′) for a heterodimer complex of the positive transcription factors Ino2p and Ino4p (11, 24, 100, 101). The role of UASINO in the regulation of *CHO1* by zinc depletion was examined using a *PCHO1*-lacZ reporter gene (100) with mutations in the cis-acting element (27). In the control experiment with the wild type reporter gene, the β-galactosidase activity from zinc-depleted cells was 40% less than that of cells grown with zinc (Fig. 4). The decrease in the reporter activity in response to zinc depletion was consistent with the results of the Northern blot analysis, indicating that transcription of *CHO1* was repressed by zinc depletion. As described previously (27), the mutations in the UASINO element caused a 43% decrease in β-galactosidase activity in wild type cells grown with zinc (Fig. 4). The β-galactosidase activity driven by the mutant reporter gene was not significantly affected by zinc depletion (Fig. 4). These results indicated that the zinc-mediated regulation of *CHO1* expression was dependent on the UASINO element in its promoter.
The growth medium for the ino2Δ, ino4Δ, and opi1Δ mutations was supplemented with inositol (75 μM). Cell extracts were prepared and assayed for PS synthase activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D.

Effect of Zinc Depletion on Phospholipid Composition in Wild Type Cells—The phospholipid composition of the wild type cells grown in the presence and absence of zinc was similar to that of wild type cells grown in the presence of zinc (Fig. 8, A and B). In particular, the amounts of PE and PI in zrt1Δ zrt2Δ mutant cells grown with zinc were reduced (66%) and elevated (28%), respectively, when compared with the amounts of PE and PI in the wild type cells grown with zinc (Fig. 8, A and B). Zinc depletion did not have a further effect on the phospholipid composition of the zrt1Δ zrt2Δ mutant with the exception of a 42% increase in PS (Fig. 8B). The phospholipid composition of the zrt3Δ (Fig. 8C) and the cot1Δ zrc1Δ (Fig. 8D) mutants grown in the presence of zinc was not significantly different from that of wild type cells grown with zinc. Like wild type cells, these mutants showed a decrease in PE and an increase in PI in response to zinc depletion (Fig. 8, C and D). These results indicated that the cytoplasmic levels of zinc that are controlled by the plasma membrane Zrt1p and Zrt2p zinc transporters were primarily responsible for regulating the cellular composition of phospholipids.

Effect of Zinc Depletion on the Expression of Aut7p—An
in the CDP-choline pathway for PC synthesis might be activated to compensate for the decrease in activities of the CDP-DAG pathway enzymes.

The elevated expression of Aut7p is a marker for the induction of autophagy (80). The increased expression of Aut7p and Aut7p-PE in response to zinc depletion indicated that this stress condition induced autophagy in yeast. Although the decrease in PE content in response to zinc depletion correlated with the induction of Aut7p and Aut7p-PE, the induction of Aut7p was not a major cause for the reduction in PE content. Therefore, in addition to the decrease in PE synthesis, cumulative effects of PE utilization by additional metabolic processes (e.g. synthesis of glycosylphosphatidylinositol anchors (3)) may have contributed to the decrease in PE content in response to zinc depletion.

One of the most highly regulated CDP-DAG pathway enzymes in *S. cerevisiae* is the CHO1-encoded PS synthase (13, 25, 123). This enzyme is regulated by genetic and biochemical mechanisms that influence the synthesis of phospholipids via the CDP-DAG pathway as well as the synthesis of PI (13, 25, 123). The expression of CHO1 is repressed by water-soluble phospholipid precursors (67, 100, 103, 104) and in the stationary phase of growth (124, 125). The PS synthase enzyme is activated by DGPP, PA, PC, and PI but inhibited by cardiolipin, diacylglycerol, sphingoid bases, inositol, and CTP (20, 34, 38, 40, 126). The enzyme is also inhibited following its phosphorylation by CAMP-dependent protein kinase (28).

We showed in this study that PS synthase was regulated by zinc availability. Zinc depletion led to the repression of CHO1 transcription, resulting in decreased levels of the PS synthase protein and activity. A lack of the UASZRE in the promoter of the CHO1 gene indicated that the transcription factor Zap1p, a zinc-regulated transcription factor (96), does not directly regulate PS synthase expression in response to zinc depletion. Moreover, an indirect effect of Zap1p on the expression of PS synthase was ruled out because the zap1Δ mutation did not affect the zinc-mediated regulation of the enzyme. Instead, the expression of PS synthase by zinc was controlled through the UASINO element and by the transcription factors Ino2p, Ino4p, and Opi1p. These regulatory components play an important role in the inositol-mediated regulation of UASINO-containing genes involved in phospholipid synthesis (13, 24, 26, 67, 100, 103). When wild type cells are grown in the presence of zinc but in the absence of inositol, the Ino2p-Ino4p complex binds the UASINO element to drive transcription for maximum gene expression (13, 24, 26, 100). When cells are grown with zinc and supplemented with inositol, Opi1p represses transcription by binding to DNA-bound Ino2p (127). Like CHO1, the UASINO-containing INO1 gene was repressed by zinc depletion although inositol was absent from the growth medium. Additional studies will be required to verify that the reduced activity levels of the PS decarboxylase, PE methyltransferase, and phospholipid methyltransferase enzymes in zinc-depleted cells were mediated through the UASINO elements present (11, 24) in the promoters of PSD1, CHO2, and OPI3, respectively. Expression of the FIS1 gene encoding PI synthase is not regulated by inositol supplementation and growth phase (33, 124), and the gene does not contain the UASZRE in its promoter. Understanding the molecular basis for the elevated expression of PI synthase activity in response to zinc depletion is a subject of future studies.

It is unclear whether the low level of CHO1 expression in response to zinc depletion resulted from reduced activation by Iln2p-Iln4p and/or by increased repression by Opi1p. Iln2p and Iln4p are not zinc-containing proteins, and thus a reduction in their function was not likely to be a direct consequence
of zinc depletion. The INO2 gene contains the UASINO element, and its expression is regulated by inositol supplementation (110). Thus, the reduced expression of CHO1 may be explained if INO2 was also repressed by zinc depletion. Data indicate that Opl1p plays the major role in the repression of UASINO-containing genes in response to inositol supplementation (128). It is unknown whether OPI1 expression is induced by zinc depletion.

2. Lipid localization (131). It is unknown whether this regulation responds to zinc depletion and stationary phase on phospholipid composition.

There are other examples of UASINO-mediated regulation of phospholipid biosynthetic genes that occurs in the absence of inositol. The UASINO-containing genes and their enzyme products are repressed when cells enter the stationary phase of growth (124, 125, 132). Cells enter this growth phase and stop proliferating when essential nutrients (e.g. carbon and nitrogen) become limiting (133). In fact, the INO1 gene is repressed by nitrogen starvation, and this regulation is mediated through its UASINO element and the transcription factor Opl1p (134). Nutrient limitation is a common stress condition in both zinc-depleted and stationary phase cells. The common effect of zinc depletion and stationary phase on phospholipid composition was an increase in PI content (124). However, in contrast to the zinc-mediated effect on PE content, stationary phase has little effect on PE (124). Thus, whereas zinc depletion and stationary phase (124, 125, 132) regulated enzyme expression through the UASINO element, these stress conditions yielded different effects on phospholipid composition.

In summary, this work showed that the expression of several phospholipid biosynthetic enzyme activities was coordinately regulated by zinc depletion. The decrease in the level of PS synthase activity was due to decreased CHO1 mRNA and protein levels. This regulation occurred through the UASINO element and by the transcription factors Ino2p, Ino4p, and Opl1p. This work demonstrates that multiple signals, other than inositol, govern the UASINO-mediated regulation of phospholipid synthesis in S. cerevisiae.

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