Regulation of the Yeast EKI1-encoded Ethanolamine Kinase by Inositol and Choline*

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Received for publication, May 21, 2004
Published, JBC Papers in Press, June 16, 2004, DOI 10.1074/jbc.M405704200

The regulation of the EKI1-encoded ethanolamine kinase by inositol and choline was examined in Saccharomyces cerevisiae. Transcription of the EKI1 gene was monitored by following the expression of β-galactosidase activity driven by a P<sub>eks</sub>-lacZ reporter gene. The addition of inositol to the growth medium resulted in a dose-dependent decrease in EKI1 expression. Supplementation of choline to inositol-containing growth medium brought about a further decrease in expression, whereas choline supplementation alone had no effect. Analysis of EKI1 expression in ino2Δ, ino4Δ, and opi1Δ mutants indicated that the transcription factors Ino2p, Ino4p, and Opi1p played a role in this regulation. Moreover, mutational analysis showed that the UAS<sub>INO</sub> element in the EKI1 promoter was required for the inositol-mediated regulation. The regulation of EKI1 expression by inositol and choline was confirmed by corresponding changes in ethanolamine kinase mRNA, protein, and activity levels. The repression of ethanolamine kinase by inositol supplementation correlated with a decrease in the incorporation of ethanolamine into CDP-ethanolamine pathway intermediates and into phosphatidylethanolamine and phosphatidylycerol.

Phosphatidylethanolamine (PE)<sup>1</sup> is the second most abundant phospholipid in cellular membranes of the yeast Saccharomyces cerevisiae (1–3). Analyses of mutants defective in the synthesis of PE have shown that this phospholipid is essential for growth when mitochondrial function is required (4, 5). PE is a non-bilayer-forming phospholipid; however, its essential role in yeast physiology is not dependent on its ability to form hexagonal phase structures (4). In addition, PE is involved in the synthesis of essential glycosylphosphatidylinositol-anchored membrane proteins (6–8) and is used directly to modify the essential autophagy protein Aut7p (9–11).

PE is synthesized by complementary pathways in S. cerevisiae (see Fig. 1) (3, 12–15). In the CDP-DAG pathway, PE is derived from CDP-DAG via phosphatidylserine. In the CDP-ethanolamine branch of the Kennedy pathway, PE is derived from exogenous ethanolamine via phosphatidylserine and CDP-ethanolamine. Phosphatidylethanolamine may also be derived from sphingolipid metabolism (13, 16, 17). The Kennedy pathway is ethanolamine kinase (ATP:ethanolamine phosphotransferase, EC 2.7.1.82) (Fig. 1). Ethanolamine kinase, which is encoded by the EKI1 gene, catalyzes the formation of phosphatidylethanolamine and ADP from ethanolamine and ATP (25). In this work, we showed that the expression of the EKI1 gene was repressed in exponential phase cells by inositol alone and in combination with choline. The phospholipid synthesis regulatory proteins Ino2p, Ino4p, and Opi1p, and the UAS<sub>INO</sub> cis-acting element in the EKI1 promoter mediated the regulation of EKI1 expression.

EXPERIMENTAL PROCEDURES

Materials

All of the chemicals were reagent grade. Growth media were from Difco. Restriction endonucleases, modifying enzymes, recombinant Vent DNA polymerase, and the NEBlot kit were from New England Biolabs. RNA size markers were from Promega. Radiochemicals, ProbeQuant G-50 columns, protein A-Sepharose<sup>™</sup> CL-4B, polyvinylidene difluoride membranes, and an enhanced chemifluorescence Western blotting detection kit were from Amersham Biosciences. Polymerase chain reaction primers were prepared by Genosys Biotechnology, Inc. The Yeastmaker™ yeast transformation system was from Clontech. Scintillation counting supplies were from National Diagnostics. Bovine serum albumin, aprotinin, benzamidine, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, inositol, choline, ethanolamine, phosphatidylethanolamine, and O-nitrophenyl β-D-galactopyranoside were purchased from Sigma. The lipids were purchased from Avanti Polar Lipids. The DNA size ladder used for agarose gel electrophoresis, Zeta Probe blotting membranes, protein assay reagents, electrophoretic reagents, immunochemical reagents, protein molecular mass standards for SDS-PAGE, and acrylamide solutions were purchased from Bio-Rad. Silica gel 60 thin layer chromatography plates were from EM Science. The QuikChange<sup>™</sup> site-directed mutagenesis

* This work was supported in part by United States Public Health Service, National Institutes of Health Grant GM-50679. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PE, phosphatidylethanolamine; PC, phosphatidylcholine; DAG, diacylglycerol.

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Methods

Strains, Plasmids, and Growth Conditions—The strains and plasmids used in this work are listed in Table 1. The methods for yeast growth were performed as described previously (26, 27). Yeast cultures were grown in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or in complete synthetic medium minus inositol (28) containing 2% glucose at 30 °C. The appropriate nutrients of complete synthetic medium were added for selection purposes.

DNA Manipulations and Amplification of DNA by PCR—Plasmid and genomic DNA preparation, restriction enzyme digestion, and DNA ligations were performed by standard methods (27). Conditions for the amplification of DNA by PCR were optimized as described previously (29). Transformation of yeast (30, 31) and E. coli (27) were performed as described previously. Plasmid maintenance and amplifications were performed in E. coli strain DH5α.

Construction of Plasmids—Plasmid pKS K10 contains the promoter sequence of the EKI1 gene fused to the coding sequence of the lacZ gene of E. coli. The plasmid was constructed by replacing the CRDI promoter in YEp357R (pSD90) with the EKI1 promoter sequence at the BamHI/EcoRI sites. The EKI1 promoter was obtained by PCR (primers, 5′- GCAGGATTCAGTTAAAACGGTCTTAAAG-3′ and 5′-TGGGAAAT- TCAGTTGAATAATGGTGTACATTATG-3′) using strain W303-1A genomic DNA as a template. The PCR primer used in the forward direction corresponds to +380 bp to the start codon, and the primer used in the reverse direction corresponds to +21 bp to the start codon. The correct orientation of the EKI1 promoter was confirmed by restriction enzyme digestion. The pKS K10 plasmid was introduced into wild type strain W303-1A to examine the expression of the EKI1 gene by measuring β-galactosidase activity. Plasmid pMC K1 is a derivative of pKS K10, in which the core sequence of the UASSp1 element (12) in the EKI1 promoter was changed from 5′-CATGCATTAAA-3′ to 5′-TTTTTT- TAAA-3′. Mutagenesis was performed with the Stratagene QuikChange™ site-directed mutagenesis kit using plasmid pKS K10 as the template and the mutagenic primers 5′ GTTGGCCACTAGA- CAGTTTTTTAACCCTGTATGAG-3′ and 5′-CTATCTACG- CGTTTAAAATGCTTACGCCCCAAC-3′. DNA sequencing confirmed the mutations in the UASSp1 sequence.

RNA Isolation and Northern Blot Analysis—Total RNA was isolated from cells using the methods of Schmitt et al. (32) and Herrick et al. (33). The RNA was resolved overnight at 22 V on a 1.1% formaldehyde gel (34) and then transferred to Zeta Probe membrane by vacuum blotting. The EKI1 and TCM probes were labeled with [γ-32P]ATP using the NEBlot random primer labeling kit, and unincorporated nucleotides were removed using ProbeQuant G-50 columns. Prehybridization, hybridization with probes, and washes to remove nonspecific binding were carried out according to the manufacturer’s instructions. Images of the radiolabeled species were acquired by phosphorimaging analysis.

Enzyme Assays and Protein Determination—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 Tris-HCl buffer (pH 7.5), 5 mM ethanolamine, 10 mM ATP, 10 mM MgSO4, and enzyme protein (0.12 mg/ml) in a final volume of 25 μl. The 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, ammonium hydroxide (10:10:1) (39). The position of the labeled phosphoethanolamine on chromatograms was visualized by phosphorimaging. The relative density of the protein was analyzed using ImageQuant software. Immunoblot signals were in the linear range of detection.

Preparation of Cell Extracts—All of the steps were performed at 5 °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 NαEdTA, 0.3 M sucrose, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidenc, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin (38). The glass beads and cell debris were removed by centrifugation at 1,500 × g for 5 min. The supernatant was used as the cell extract.

Thin Layer Chromatography—Phospholipids were analyzed by thin layer chromatography with potassium oxalate-impregnated silica gel plates using the solvent system of methanol, 0.6% sodium chloride, ammonium hydroxide (10:10:1) (39). The position of the labeled phosphoethanolamine on chromatograms was visualized by phosphorimaging and compared with a phosphoethanolamine standard. The amount of labeled product was determined by scintillation counting. β-Galactosidase activity was determined using the conversion of O-nitrophenyl β-D-galactopyranoside to O-nitrophenol (molar extinction coefficient of 3,500 M–1 cm–1) by following the increase in absorbance at 400 nm. A recording spectrophotometer (41) was used. The reaction mixtures 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 of the assays were performed in triplicate and were linear with time and protein concentration. Specific activity was defined as units/mg of protein. Protein concentration was determined by the method of Bradford (41) using bovine serum albumin as the standard.

Western Blotting Detection Kit—The antibody was a generous gift from John Dyer (42). The amount of antibody was determined by liquid scintillation counting.

Affinity Chromatography—Phospholipids were extracted from whole cells by the method of Bligh and Dyer (42) as described previously (43). Phospholipids were analyzed by thin layer chromatography with potassium oxalate-impregnated silica.
constitutive low expression of the INO1 and ino4 factors Ino2p and Ino4p, respectively. Because the inositol in the presence or absence of inositol (Fig. 4). However, transcription of nolamine can also enhance the repressive effect of inositol (22, 44). For some phospholipid biosynthetic genes, ethanolamine (Fig. 3). Choline had no effect on expression when compared with cells grown in the absence of grown only in the presence of inositol and a 63% reduction in activity when compared with cells grown in the absence of inositol. The level used for wild type cells was 0.04 unit/mg. Each data point represents the average of triplicate determinations from two independent experiments ± S.D.

For many phospholipid biosynthetic genes, the repressive effect of inositol is enhanced by the inclusion of choline to the growth medium (12, 13, 22). Accordingly, we questioned whether choline had an effect on the inositol-mediated regulation of EKI1. Wild type cells containing the P_EKI1-lacZ reporter gene were grown to the exponential phase of growth in the absence of inositol and choline (12, 22, 47). The element contains a consensus-binding site (5′-CANNTG-3′) for a heterodimer complex of the positive transcription factors Ino2p and Ino4p, which are also required for maximum expression in growth medium not supplemented with inositol and choline (12, 22, 47, 48). Because the promoter region of the EKI1 gene contains a consensus sequence for the UASINO cis-acting element (25), we questioned whether this sequence played a role in the transcriptional

**RESULTS**

Effect of Inositol and Choline on the Expression of β-Galactosidase Activity in Cells Containing the P_EKI1-lacZ Reporter Gene—A P_EKI1-lacZ reporter gene was used to study the transcriptional regulation of the EKI1 gene. The P_EKI1-lacZ reporter gene was constructed by fusing the EKI1 promoter in frame with the coding sequence of the E. coli lacZ gene. Therefore, the expression of β-galactosidase activity was dependent on transcription driven by the EKI1 promoter. Wild type cells containing the P_EKI1-lacZ reporter gene were grown to the exponential phase of growth in the absence or presence of various concentrations of inositol. Cell extracts were then prepared and used for the assay of β-galactosidase activity. The addition of inositol to the growth medium resulted in a dose-dependent decrease in β-galactosidase activity (Fig. 2). Maximum repression of β-galactosidase activity (37%) occurred when cells were grown with 40–60 μM inositol.

For many phospholipid biosynthetic genes, the repressive effect of inositol is enhanced by the inclusion of choline to the growth medium (12, 13, 22). Accordingly, we questioned whether choline had an effect on the inositol-mediated regulation of EKI1. Wild type cells containing the P_EKI1-lacZ reporter gene were grown to the exponential phase of growth in the absence of inositol and choline (12, 22, 47). The element contains a consensus-binding site (5′-CANNTG-3′) for a heterodimer complex of the positive transcription factors Ino2p and Ino4p, which are also required for maximum expression in growth medium not supplemented with inositol and choline (12, 22, 47, 48). Because the promoter region of the EKI1 gene contains a consensus sequence for the UASINO cis-acting element (25), we questioned whether this sequence played a role in the transcriptional

Effect of inositol supplementation on the expression of β-galactosidase activity in wild type cells bearing the P_EKI1-lacZ reporter gene. Wild type cells bearing the P_EKI1-lacZ reporter plasmid pSK10 were grown to the exponential phase of growth in the absence and presence of the indicated concentrations of inositol. The cell extracts were prepared and assayed for β-galactosidase activity. The specific activity of β-galactosidase from cells grown in the absence of inositol was 0.04 unit/mg. Each data point represents the average of triplicate determinations from two independent experiments ± S.D.

**FIG. 2.** Effect of inositol supplementation on the expression of β-galactosidase activity in wild type cells bearing the P_EKI1-lacZ reporter gene. Wild type cells bearing the P_EKI1-lacZ reporter plasmid pSK10 were grown to the exponential phase of growth in the absence and presence of the indicated concentrations of inositol. The cell extracts were prepared and assayed for β-galactosidase activity. The specific activity of β-galactosidase from cells grown in the absence of inositol was 0.04 unit/mg. Each data point represents the average of triplicate determinations from two independent experiments ± S.D.

**TABLE I**

<table>
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<th>Strain or plasmid</th>
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<td>pKSK3</td>
<td>EKI1 gene on a multicopy plasmid with URA3, derivative of YEp352</td>
<td>Ref. 25</td>
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regulation of EKI1 by the phospholipid precursors. The UASINO element in the P_EKI1-lacZ reporter gene was mutated to a nonconsensus sequence for the Ino2p-Ino4p heterodimer-binding site. The mutations in the UASINO element caused a 77% decrease in β-galactosidase activity in cells grown without supplementation when compared with cells with the wild type promoter (Fig. 6). In addition, the β-galactosidase driven by the mutant reporter gene was not repressed by supplementation of inositol alone and in combination with choline (Fig. 6).

Effects of Inositol and Choline on the Expression of Ethanolamine Kinase mRNA, Protein, and Activity Levels—We carried out experiments to show that the expression of the EKI1 gene products was regulated in response to inositol and choline. For these studies, the EKI1 gene was expressed on a multicopy plasmid in the cki1Δ eki1Δ double mutant. The multicopy plasmid was used because the EKI1 was expressed at very low levels in wild type cells. EKI1 expression was examined in a cki1Δ mutant background to obviate interference from the CKI1 gene product that exhibits some ethanolamine kinase activity (49). We examined the levels of EKI1 mRNA by Northern blot analysis of total RNA extracted from cells grown in the absence and presence of inositol and choline. The expression of TCM1 mRNA served as a loading control. The TCM1 gene encodes a ribosomal protein that is not regulated by inositol supplementation (46, 50). Supplementation of inositol to the growth medium resulted in a decrease (23%) in the relative abundance of EKI1 mRNA (Fig. 7). The addition of choline to inositol-containing medium resulted in a further decrease (44%) in EKI1 mRNA expression (Fig. 7). Choline alone did not affect the level of the EKI1 transcript (Fig. 7).

The levels of the ethanolamine kinase protein (Eki1p) were compared by immunoblot analysis of cell extracts derived from cki1Δ eki1Δ mutant cells bearing the EKI1 gene that were grown in the absence and presence of inositol and choline. Antibodies generated against a peptide sequence at the C-terminal end of Eki1p recognized ethanolamine kinase present in cell extracts (Fig. 8). The specificity of the reaction was confirmed using cell extracts derived from the cki1Δ eki1Δ double mutant (Fig. 8). Inositol supplementation resulted in a 30% decrease in the level of the ethanolamine kinase protein when compared with that of cells grown in the absence of inositol (Fig. 8). The addition of choline to inositol-containing medium resulted in a 50% decrease in the ethanolamine kinase protein when compared with cells without any supplementation (Fig. 8). The levels of the ethanolamine kinase protein were not affected by choline when inositol was absent from the growth medium (Fig. 8).

The results of the Northern and immunoblot experiments suggested that the expression of ethanolamine kinase activity should be regulated in response to inositol and choline. Accordingly, ethanolamine kinase activity was measured in cell extracts derived from cki1Δ eki1Δ mutant cells bearing the EKI1 gene. The addition of inositol to the growth medium resulted in a 35% reduction in ethanolamine kinase activity when com-
under and phospholipids were extracted and analyzed as described under Experimental Procedures. The amount of PE in inositol-supplemented cells was reduced when compared with cells grown without inositol (Fig. 10). The [1,2-14C]ethanolamine label was also incorporated by 70%. The [1,2-14C]ethanolamine label was also incorporated by 70%. The effect of inositol on the composition of the CDP-ethanolamine pathway intermediates and the phospholipids PE and PC. Wild type cells were grown to the exponential phase of growth in the absence and presence of the indicated combinations of 50 μM inositol and 50 μM choline. The specific activity of ethanolamine kinase from cells grown without supplementation was 3.38 units/mg. Each data point represents the average of triplicate determinations from two independent experiments ± S.D.

FIG. 7. Effects of inositol and choline on the abundance of EKI1 mRNA. cklΔckiΔ mutant cells bearing plasmid pKSK3, which contains the EKI1 gene, were grown to the exponential phase of growth in the absence and presence of the indicated combinations of 50 μM inositol and 50 μM choline. Total RNA was extracted, and the abundance of EKI1 mRNA was determined with 25 μg of RNA by Northern blot analysis as described under "Experimental Procedures." Relative amounts of the EKI1 transcript were determined by ImageQuant analysis. The amount of EKI1 mRNA found in cells grown without inositol and choline was set at 100%. The data shown are representative of two independent experiments.

FIG. 8. Effects of inositol and choline on the levels of ethanolamine kinase protein. cklΔckiΔ mutant cells bearing plasmid pKSK3, which contains the EKI1 gene, were grown to the exponential phase of growth in the absence and presence of the indicated combinations of 50 μM inositol and 50 μM choline. The cell extracts (30 μg of protein) were subjected to immunoblot analysis using a 1 μg/ml dilution of a purified IgG fraction from anti-ethanolamine kinase antibodies. A portion of the immunoblot is shown, and the position of the 60-kDa ethanolamine kinase protein (Eki1p) is indicated. Relative amounts of Eki1p were determined by ImageQuant analysis.

FIG. 9. Effects of inositol and choline on the expression of ethanolamine kinase activity. cklΔckiΔ mutant cells bearing plasmid pKSK3, which contains the EKI1 gene, were grown to the exponential phase of growth in the absence and presence of the indicated combinations of 50 μM inositol and 50 μM choline. The cell extracts were prepared and assayed for ethanolamine kinase activity. The specific activity of ethanolamine kinase from cells grown without supplementation was 3.38 units/mg. Each data point represents the average of triplicate determinations from two independent experiments ± S.D.

FIG. 10. Effect of inositol on the composition of the CDP-ethanolamine pathway intermediates and the phospholipids PE and PC. Wild type cells were grown to the exponential phase of growth in the absence or presence of 50 μM inositol (I). The 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 and analyzed as described under "Experimental Procedures." The values reported were the average of three separate experiments ± S.D. Etn, ethanolamine; P-Etn, phosphoethanolamine; CDP-Etn, CDP-ethanolamine.

The uptake was nearly abolished by the presence of choline in the growth medium (data not shown).

DISCUSSION

PE, the second most abundant phospholipid in S. cerevisiae, is synthesized by the complementary CDP-DAG and Kennedy (CDP-ethanolamine branch) pathways (1–3). Understanding the regulation of PE synthesis is important because it plays an essential role in yeast physiology when cells are grown with nonfermentable carbon sources (4, 5). The importance of PE in cell physiology extends to higher eukaryotes. In Drosophila melanogaster, PE controls release of the sterol regulatory element-binding protein from cell membranes to exert feedback control on the synthesis of fatty acids and phospholipids (51). In mammalian cells, PE plays an essential role in cytokinesis (52). Although a great deal is known about the regulation of PE synthesis via the CDP-DAG pathway, little information is available on the control of the CDP-ethanolamine pathway. The EKI1-encoded ethanolamine kinase (25) should play an important regulatory role in PE synthesis because the enzyme
catalyzes the committed step in the CDP-ethanolamine pathway (1–3). Indeed, a mutation in the \textit{eas} gene encoding ethanolamine kinase in \textit{D. melanogaster} results in seizure, neurological failure, and paralysis, phenotypes attributed to a defect in the synthesis of PE via the CDP-ethanolamine pathway (53). A large number of genes encoding phospholipid biosynthetic enzymes in \textit{S. cerevisiae} are regulated by the inclusion of inositol and choline in the growth medium (3, 12, 13, 22, 23). This regulation occurs at the transcriptional level and is due to the presence of the UAS\textsubscript{INO} cis-acting element present in the promoter regions of their genes (12, 22, 47). The transcripts of the genes containing the UAS\textsubscript{INO} element are maximally expressed during exponential growth in medium lacking inositol and choline. The inclusion of inositol and choline in the growth medium represses the expression of these genes (3, 12, 13, 22–24). The \textit{EKI1} gene was shown to contain a UAS\textsubscript{INO} element in its promoter sequence (25); however, not all UAS\textsubscript{INO}-containing genes encoding phospholipid biosynthetic enzymes are regulated by inositol and choline. For example, the \textit{PIS1} gene, which encodes phosphatidylinositol synthase, contains a UAS\textsubscript{INO} element in its promoter sequence, but its expression is not regulated by inositol alone or in combination with choline or ethanolamine (22, 44, 54, 55). In this work, we showed that expression of the \textit{EKI1}-encoded ethanolamine kinase was indeed regulated by inositol and choline. Maximum expression of the \textit{EKI1} gene, as monitored by the \(\beta\)-galactosidase activity driven by the \textit{P\_EKI1}-lacZ reporter gene, occurred when wild-type cells were grown in the absence of phospholipid precursors. This level of expression was dependent on the UAS\textsubscript{INO} element in the \textit{EKI1} promoter and the positive transcription factors Ino2p and Ino4p. This conclusion was supported by the reduced levels of \textit{P\_EKI1}-lacZ-driven \(\beta\)-galactosidase activity in wild-type cells bearing the reporter gene with mutations in the UAS\textsubscript{INO} element and by the reduced \(\beta\)-galactosidase activity in the \textit{ino2} and \textit{ino4} mutants.

Inositol supplementation resulted in the repression of \textit{EKI1} expression, and this regulation was enhanced by choline but not by ethanolamine. Repression of \textit{P\_EKI1}-lacZ-driven \(\beta\)-galactosidase activity by inositol alone and in combination with choline was abolished in wild-type cells bearing the reporter gene with mutations in the UAS\textsubscript{INO} element and in the \textit{ino2} and \textit{ino4} mutants. These results supported the conclusion that Ino2p, Ino4p, and the UAS\textsubscript{INO} element in the \textit{EKI1} promoter played a role in \textit{EKI1} repression by inositol and choline. Repression of UAS\textsubscript{INO}-containing phospholipid biosynthetic genes by inositol and choline is dependent on the negative transcription factor Opi1p (3, 12, 13, 22, 23). Opi1p mediates its negative regulatory role through the UAS\textsubscript{INO} element (56) but not by direct interaction (57). In \textit{vitro} data indicate that Opi1p represses transcription by binding to DNA-bound Ino2p (58).

That the expression of \textit{EKI1} in the \textit{opi1} mutant was elevated in cells grown without supplementation and this expression was not repressed by supplementation with inositol and choline indicated that Opi1p played a negative regulatory role in \textit{EKI1} expression. Based on studies with the \textit{INO1} (59, 60) and the \textit{CHO1} (47) promoters, we propose that a heterodimer of Ino2p-Ino4p binds the UAS\textsubscript{INO} element in the \textit{EKI1} promoter to drive maximum expression and Opi1p represses this expression.

The transcriptional regulation of \textit{EKI1} by inositol and choline was confirmed by the expression of \textit{EKI1} mRNA abundance and the levels of ethanolamine kinase protein and activity. Because of the low level of \textit{EKI1} expression, it was difficult to measure and quantify changes in \textit{EKI1} mRNA and ethanolamine kinase protein levels in response to inositol and choline in wild-type cells. Accordingly, regulation studies were carried out with the \textit{EKI1} gene on a multicopy plasmid in the \textit{eki1Δ} mutant background. Ethanolamine kinase activity measurements in wild-type cells indicated that the overexpression of \textit{EKI1} did not alter the general pattern of regulation in response to inositol and choline supplementation.

The major effects of inositol supplementation on phospholipid composition of wild-type cells include a 2–3-fold increase in phosphatidylinositol content and about a 2-fold decrease in phosphatidylcholine content (2, 3). These changes have been largely attributed to the genetic and biochemical regulation of the CDP-DAG-dependent enzymes phosphatidylinositol synthase and phosphatidylserine synthase (61). In this study, the ethanolamine-labeling experiments showed that the inositol-mediated regulation of \textit{EKI1} expression correlated with a significant decrease in PE synthesis via the CDP-ethanolamine branch of the Kennedy pathway. This was reflected in decreases in the levels of the CDP-ethanolamine pathway intermediates (phosphoethanolamine and CDP-ethanolamine) as well as a decrease in PE. PC, which was derived from the methylation of PE synthesized by the CDP-ethanolamine pathway, was also reduced in response to inositol supplementation. Although the combination of inositol and choline brought about the most dramatic reduction in \textit{EKI1} expression, the effects of choline on PE synthesis via the CDP-ethanolamine pathway could not be determined in our studies because choline inhibited the uptake of [1,2-\(^{14}\)C]ethanolamine. This can be attributed to the choline-mediated repression of the \textit{HNM1}-encoded choline/ethanolamine transporter (62, 63).

In \textit{vitro} studies have shown that the \textit{EPT1}-encoded ethanolamine phosphotransferase and the \textit{CPT1}-encoded choline phosphotransferase enzymes (CDP-ethanolamine and CDP-choline branches, respectively; Fig. 1) have distinct preferences for the molecular species of DAG used for the synthesis of PE and PC, respectively (64). For example, the ethanolamine phosphotransferase shows the greatest activity with di-unsaturated DAG species (64). This suggests that the molecular species of PC made through the CDP-choline pathway differs from the molecular species of PC made through the methylation of PE that is produced from the CDP-ethanolamine pathway. Data also indicate that the PC synthesized via the CDP-DAG and Kennedy (CDP-choline branch) pathways is not functionally equivalent (65, 66). The two pathways appear to yield PC with different molecular species needed for different membrane functions (66). It is unknown whether the PE synthesized by the CDP-DAG and CDP-ethanolamine pathways have different cellular functions. Nonetheless, the regulation of the \textit{EKI1}-encoded ethanolamine kinase by inositol supplementation must contribute to the relative levels of PE molecular species as well as the PC molecular species produced in the cell.

Acknowledgments—We thank Keunsung Kim for the construction of the \textit{P\_EKI1}-lacZ reporter gene, Susan A. Henry for providing us with the \textit{ino2, ino4}, and \textit{opi1} mutants, and William Dowhan for plasmid pSSD90. We also acknowledge Avula Sreenivas, Gil-Soo Han, and Wendy Iwanyshyn for helpful discussions.

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