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

Michael C. Kersting, Hyeon-Son Choi, and George M. Carman

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

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 aPES-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 UASINO 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 phosphatidylcholine.

Phosphatidylethanolamine (PE) 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-ethanolamine via phosphatidylserine. In the CDP-DAG pathway, PE is derived from exogenous ethanolamine via phosphoethanolamine and ADP from ethanolamine and ATP (25). Ethanolamine kinase, which catalyzes the committed step in the synthesis of phosphatidylinositol (INP1) and PC (CD51, CHO1/PSS1, PSD1, CHO2/PEM1, OPI3/PEM2, CKI1, and CPT1) is regulated by inositol (3, 12, 13, 22–24). These genes are maximally expressed when inositol is absent from the growth medium and repressed when inositol is added to the growth medium. Repression by inositol is enhanced by choline or ethanolamine supplementation (3, 12, 13, 22, 23). In addition, these genes are maximally expressed in the exponential phase and are repressed in the stationary phase (13, 22, 23). This growth phase-mediated regulation is independent of the presence of inositol in the growth medium (13, 22, 23).

There is a lack of information regarding the regulation of the expression of the genes encoding enzymes responsible for PE synthesis via the CDP-ethanolamine branch of the Kennedy pathway. The enzyme that catalyzes the committed step in this pathway is ethanolamine kinase (ATP:ethanolamine phospho-transferase, 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 UASINO 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, Probe-Quant G-50 columns, protein A-Sepharose™ CL-4B, polyvinylidene difluoride membranes, and an enhanced chemiluminescence 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, phosphatidylcholine, CDP-ethanolamine, 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, immunochromagenic 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™ site-directed mutagenesis
EcoRI sites. The EKI1 gene in YEp357R (pSD90) with the EKI1 sequence of the genomic DNA as a template. The PCR primer used in the forward direction corresponds to the genomic DNA preparation, restriction enzyme digestion, and DNA gel electrophoresis. The plasmid was constructed by replacing the core sequence of the UAS INO element (12) with the EKI1 promoter sequence at the BamHI/EcoRI sites. The EKI1 promoter was obtained by PCR (primers, 5'-GCAGGATCCAGAGTTAAGCGTTCAGG-3' and 5'-TGGAATTCAGTTAATACGTGTTACATGTCG-3') using strain W303-1A genomic DNA as a template. The PCR primer used in the forward direction corresponds to the EcoRI site and the primer used in the reverse direction corresponds to the C-terminal end of the deduced protein sequence of EKI1.

Construction of Plasmids—Plasmid pKSK10 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 YEps57R (pSD90) with the EKI1 promoter sequence at the BamHI/EcoRI sites. The EKI1 promoter was obtained by PCR (primers, 5'-GCAGGATCCAGAGTTAAGCGTTCAGG-3' and 5'-TGGAATTCAGTTAATACGTGTTACATGTCG-3') using strain W303-1A genomic DNA as a template. The PCR primer used in the forward direction corresponds to the EcoRI site and the primer used in the reverse direction corresponds to the C-terminal end of the deduced protein sequence of EKI1.

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, 28). 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.

Enzyme Assays and Protein Determination—Ethanolamine kinase activity was measured for 40 min at 30 °C by following the phosphorylation of [1,2-3H]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 fluorescent imaging. The relative density of the protein was analyzed using ImageQuant software. Immunoblot signals were in the linear range of detectability.

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 Na2EDTA, 0.3 mM 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 (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.

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% agarose gel (34) and then transferred to Zeta Probe membrane by vacuum blotting. The EKI1 and TCMI probes were labeled with [α-32P]dCTP 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 non-specific binding were carried out according to the manufacturer's instructions. Images of the radiolabeled species were acquired by phosphoimaging analysis.
Regulation of the Yeast Ethanolamine Kinase

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 and 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 inositol-containing medium in the presence of various concentrations of choline. The addition of choline to the growth medium resulted in a dose-dependent decrease in the level of β-galactosidase activity (Fig. 3). The maximum level of repression occurred in cells grown in the presence of 50 μM choline. This resulted in a 23% further reduction in activity when compared with cells grown only in the presence of inositol and a 63% reduction in expression when compared with cells grown in the absence of inositol and choline (Fig. 3). Choline had no effect on EKI1 transcription when inositol was absent from the growth medium (Fig. 4). For some phospholipid biosynthetic genes, ethanolamine can also enhance the repressive effect of inositol (22, 44). However, transcription of EKI1 was not affected by ethanolamine in the presence or absence of inositol (Fig. 4).

Roles of Ino2p, Ino4p, and Opi1p, and the UASINO cis-acting element in the regulation of EKI1 by inositol and choline—The transcriptional regulation of EKI1 (as monitored by P_{EKI1}-lacZ reporter gene activity) by inositol and choline was examined in ino2Δ and ino4Δ mutants defective in the positive transcription factors Ino2p and Ino4p, respectively. Because the ino2Δ and ino4Δ mutants are inositol auxotrophs (45), because of the constitutive low expression of the INO1 gene (46), it was necessary to supplement their growth medium with 10 μM inositol. This growth condition was considered to be analogous to that of wild type cells not supplemented with inositol (46). An inositol concentration of 60 μM was added to the growth medium of the ino2Δ and ino4Δ mutants to mimic the inositol-supplemented level used for wild type cells. The β-galactosidase activity in the ino2Δ and ino4Δ mutants grown with 10 μM inositol was reduced by 37 and 30%, respectively, when compared with the activity of wild type cells grown without inositol (Fig. 5). In contrast to wild type cells, the addition of inositol alone and in combination with choline did not result in the repression of β-galactosidase activity (Fig. 5). Regulation of EKI1 was also examined in the opi1Δ mutant defective in the negative transcription factor Opi1p. The β-galactosidase driven by the P_{EKI1}-lacZ reporter gene in the opi1Δ mutant grown with or without supplementation was elevated by 30% when compared with wild type cells (Fig. 5). Moreover, inositol supplementation with and without choline did not have a major effect on the expression of β-galactosidase activity (Fig. 5).

A UASINO cis-acting element in the promoter of several phospholipid biosynthetic genes is required for their maximum expression when wild type cells are grown 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 element (25), we questioned whether this sequence played a role in the transcriptional

---

Table 1

| Strain or plasmid | Genotype or relevant characteristics | Source or reference |
|-------------------|-------------------------------------|---------------------|
| **S. cerevisiae strains** | | |
| W303-1A | MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 | Ref. 67 |
| W303-1B | MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 | Ref. 67 |
| K106 | chs1::HIS3 411Gln-TRP1 derivative of W303-1B | Ref. 25 |
| SH303 | MATa his3Δ200 leu2Δ1 trp1Δ363 ura3-52 ino2Δ::LEU2 | S. A. Henry |
| SH307 | MATa his3Δ200 leu2Δ1 trp1Δ363 ura3-52 ino4Δ::LEU2 | S. A. Henry |
| SH304 | MATa his3Δ200 leu2Δ1 trp1Δ363 ura3-52 opi1Δ::LEU2 | S. A. Henry |
| **E. coli strains** | | |
| DH5α | F- d800lacZAM15 ΔlacZYA-argF U169 recA1 endA1 hsdR17(rk- mK+) supE44 | Ref. 27 |
| **Plasmids** | | |
| pSD90 | P_{crd1}-lacZ reporter gene containing the CRD1 promoter with URA3, derivative of pMA109 | W. Dowhan |
| pKSK10 | P_{EKI1}-lacZ reporter gene containing the EKI1 promoter with URA3, derivative of pSD90 | This work |
| pMCK1 | Derivative of pKSK10 containing the EKI1 promoter with mutations in the UASINO element | This work |
| pKK3 | EKI1 gene on a multicopy plasmid with URA3, derivative of YEp352 | Ref. 25 |

---

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 pKSK10 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. 3. Effect of choline on the expression of β-galactosidase activity in inositol-supplemented wild type cells bearing the P\textsuperscript{EKI1}-lacZ reporter gene. Wild type cells bearing the P\textsuperscript{EKI1}-lacZ reporter plasmid pKSK10 were grown to the exponential phase of growth with 50 μM inositol in the absence and presence of the indicated concentrations of choline. The cell extracts were prepared and assayed for β-galactosidase activity. The specific activity of β-galactosidase from cells grown in the absence of choline but in the presence of 50 μM choline was 0.025 unit/mg. The percentages shown in the figure were relative to the activity from cells grown in the absence of inositol and choline. Each data point represents the average of triplicate determinations from two independent experiments ± S.D.

FIG.4. Effects of inositol, choline, and ethanolamine on the expression of β-galactosidase activity in wild type cells bearing the P\textsuperscript{EKI1}-lacZ reporter gene. Wild type cells bearing the P\textsuperscript{EKI1}-lacZ reporter plasmid pKSK10 were grown to the exponential phase of growth in the absence and presence of the indicated combinations of 50 μM inositol, 50 μM choline, and 50 μM ethanolamine. The cell extracts were prepared and assayed for β-galactosidase activity. The specific activity of β-galactosidase from cells grown without supplementation was 0.04 unit/mg. Each data point represents the average of triplicate determinations from two independent experiments ± S.D.

FIG.5. Effects of the ino2Δ, ino4Δ, and opi1Δ mutations on the expression of the EKI1 gene in response to inositol and choline. Wild type (WT), ino2Δ, ino4Δ, and opi1Δ cells bearing the P\textsuperscript{EKI1}-lacZ reporter plasmid pKSK10 were grown to the exponential phase of growth in the absence or presence of the indicated combinations of 50 μM inositol (I) and 50 μM choline (C). The cell extracts were prepared and assayed for β-galactosidase activity. The specific activity of β-galactosidase from cells grown without supplementation was 0.05 unit/mg. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D.

Fig. 4. Effect of UAS\textsubscript{INO} mutations on the regulation of the EKI1 gene by inositol and choline. Wild type cells bearing the P\textsuperscript{EKI1}-lacZ reporter plasmid pKSK10 or pMCK1 were grown in the absence and presence of the indicated combinations of 50 μM inositol (I) and 50 μM choline (C). The cell extracts were prepared and used for the assay of β-galactosidase activity. The specific activity of β-galactosidase from cells grown without supplementation was 0.04 unit/mg. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D. Mutations in the UAS\textsubscript{INO} element in plasmid pMCK1 are underlined.

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 TCMI mRNA served as a loading control. The TCMI 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-

regulation of EKI1 by the phospholipid precursors. The UAS\textsubscript{INO} element in the P\textsuperscript{EKI1}-lacZ reporter gene was mutated to a nonconsensus sequence for the Ino2p-Ino4p heterodimer-binding site. The mutations in the UAS\textsubscript{INO} 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).

FIG.6. Effect of UAS\textsubscript{INO} mutations on the regulation of the EKI1 gene by inositol and choline. Wild type cells bearing the P\textsuperscript{EKI1}-lacZ reporter plasmid pKSK10 or pMCK1 were grown in the absence and presence of the indicated combinations of 50 μM inositol (I) and 50 μM choline (C). The cell extracts were prepared and used for the assay of β-galactosidase activity. The specific activity of β-galactosidase from cells grown without supplementation was 0.04 unit/mg. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D. Mutations in the UAS\textsubscript{INO} element in plasmid pMCK1 are underlined.
and phospholipids were extracted and analyzed 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.

The regulation by choline was dependent on the presence of inositol in the growth medium (Fig. 9).

**Effect of Inositol on the Composition of the CDP-Ethanolamine Pathway Intermediates, PE, and PC**—To examine the effects of inositol supplementation on PE synthesized via the CDP-ethanolamine branch of the Kennedy pathway, wild type 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 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.

**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 eas gene encoding ethanolamine kinase in *D. melanogaster* results in seizure, neuronal 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 *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 UASINO cis-acting element present in the promoter regions of their genes (12, 22, 47). The transcripts of the genes containing the UASINO 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 EKI1 gene was shown to contain a UASINO element in its promoter sequence (25); however, not all UASINO-containing genes encoding phospholipid biosynthetic enzymes are regulated by inositol and choline. For example, the PIS1 gene, which encodes phosphatidylinositol synthase, contains a UASINO 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 EKI1-encoded ethanolamine kinase was indeed regulated by inositol and choline. Maximum expression of the EKI1 gene, as monitored by the β-galactosidase activity driven by the 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 UASINO element in the EKI1 promoter and the positive transcription factors Ino2p and Ino4p. This conclusion was supported by the reduced levels of P_{EKI1-}lacZ-driven β-galactosidase activity in wild type cells bearing the reporter gene with mutations in the UASINO element and by the reduced β-galactosidase activity in the ino2Δ and ino4Δ mutants.

Inositol supplementation resulted in the repression of EKI1 expression, and this regulation was enhanced by choline but not by ethanolamine. Repression of P_{EKI1-}lacZ-driven β-galactosidase activity by inositol alone and in combination with choline was abolished in wild type cells bearing the reporter gene with mutations in the UASINO element and in the ino2Δ and ino4Δ mutants. These results supported the conclusion that Ino2p, Ino4p, and the UASINO element in the EKI1 promoter played a role in EKI1 repression by inositol and choline. Repression of UASINO-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 UASINO element (56) but not by direct interaction (57). In vitro data indicate that Opi1p represses transcription by binding to DNA-bound Ino2p (58).

That the expression of EKI1 in the opi1Δ 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 EKI1 expression. Based on studies with the INO1 (59, 60) and the CHO1 (47) promoters, we propose that a heterodimer of Ino2p-Ino4p binds the UASINO element in the EKI1 promoter to drive maximum expression and Opi1p represses this expression.

The transcriptional regulation of EKI1 by inositol and choline was confirmed by the expression of EKI1 mRNA abundance and the levels of ethanolamine kinase protein and activity. Because of the low level of EKI1 expression, it was difficult to measure and quantify changes in 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 EKI1 gene on a multicopy plasmid in the *cki1Δ* mutant background. Ethanolamine kinase activity measurements in wild type cells indicated that the overexpression of 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 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 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 [1,2-14C]ethanolamine. This can be attributed to the choline-mediated repression of the HNM1-encoded choline/ethanolamine transporter (62, 63).

In vitro studies have shown that the EPT1-encoded ethanolamine phosphotransferase and the 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 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 P_{EKI1-}lacZ reporter gene, Susan A. Henry for providing us with the ino2Δ, ino4Δ, and opi1Δ mutants, and William Dowhan for plasmid pSSD90. We also acknowledge Avula Sreenivas, Gil-Soo Han, and Wendy Iwanyshyn for helpful discussions.

REFERENCES
1. Ratner, J. B., Schiebel, A., and Kidley, D. K. (1975) *Bacteriol. Rev.* 39, 197–231
2. Henry, S. A. (1982) in *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, NY
3. Paltau, F., Kahlwein, S. D., and Henry, S. A. (1992) in *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. Streyer, M. K., Clay, K. L., Kutateladze, T., Murphy, R. C., Overduin, M., and Reinhart, D. R. (2001) *J. Biol. Chem.* 276, 48509–48518
5. Birner, R., Burgermeister, M., Schneider, R., and Daum, G. (2001) *Mol. Biol. Cell* 12, 997–1007
6. Menon, A. K., and Stevens, V. L. (1992) *J. Biol. Chem.* 267, 15277–15280
7. Hong, Y., Maeda, Y., Watanabe, R., Oishi, K., Mishkind, M., Riehm, E., and Kinoshita, T. (1999) *J. Biol. Chem.* 274, 35099–35106
8. Benachour, A., Sipos, G., Flury, I., Reggiori, F., Canivene-Gansel, E., Vinnet, M. Birner, R., Burgermeister, M., Schneiter, R., and Daum, G. (2001) *Mol. Biol. Cell* 12, 997–1007
