Isolation and Characterization of the *Saccharomyces cerevisiae* EKI1 Gene Encoding Ethanolamine Kinase

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Ethanolamine kinase (ATP:ethanolamine O-phosphotransferase, EC 2.7.1.82) catalyzes the committed step of phosphatidylethanolamine synthesis via the CDP-ethanolamine pathway. The gene encoding ethanolamine kinase (*EKI1*) was identified from the *Saccharomyces Genome Data Base* (locus YDR147W) based on its homology to the *Saccharomyces cerevisiae* *CKI1*-encoded choline kinase, which also exhibits ethanolamine kinase activity. The *EKI1* gene was isolated and used to construct *eki1A* and *eki1Δ* *cki1A* mutants. A multicopy plasmid containing the *EKI1* gene directed the overexpression of ethanolamine kinase activity in wild-type, *eki1A* mutant, *cki1A* mutant, and *eki1Δ* *cki1A* double mutant cells. The heterologous expression of the *S. cerevisiae* *EKI1* gene in *Sf9* insect cells resulted in a 165,500-fold overexpression of ethanolamine kinase activity relative to control insect cells. The *EKI1* gene product also exhibited choline kinase activity. Biochemical analyses of the enzyme expressed in insect cells, in *eki1A* mutants, and in *cki1A* mutants indicated that ethanolamine was the preferred substrate. The *eki1A* mutant did not exhibit a growth phenotype. Biochemical analyses of *eki1A*, *cki1A*, and *eki1Δ* *cki1A* mutants showed that the *EKI1* and *CKI1* gene products encoded all of the ethanolamine kinase and choline kinase activities in *S. cerevisiae*. In *vivo* labeling experiments showed that the *EKI1* and *CKI1* gene products had overlapping functions with respect to phospholipid synthesis. Whereas the *EKI1* gene product was primarily responsible for phosphatidylethanolamine synthesis via the CDP-ethanolamine pathway, the *CKI1* gene product was primarily responsible for phosphatidylcholine synthesis via the CDP-choline pathway. Unlike *cki1A* mutants, *eki1Δ* mutants did not suppress the essential function of *Sec14p*.

The major membrane phospholipids in the yeast *Saccharomyces cerevisiae* are PC, PE, PI, and PS (1, 2). In addition to being major structural components of cellular membranes, they play important roles in various cellular processes including signal transduction (1, 2). Nearly all of the genes encoding the enzymes responsible for the synthesis of these phospholipids have been isolated and characterized, and many of the enzymes have been purified and studied (1, 2). The pathways for the synthesis of the major phospholipids in *S. cerevisiae* are shown in Fig. 1. Two alternative pathways, the CDP-DAG pathway and the CDP-choline pathway (Fig. 1), synthesize PC, the most abundant membrane phospholipid. Wild-type cells primarily use the CDP-DAG pathway when they are grown in the absence of choline (1, 2). The CDP-choline pathway becomes essential for PC synthesis when the enzymes in the CDP-DAG pathway are defective (1, 2). Mutants defective in the synthesis of PS (3, 4), PE (5, 6), or PC (7–10) are auxotrophic for choline. The choline is transported into these mutant cells and used to synthesize PC via the CDP-choline pathway. Mutants defective in the synthesis of PS (3, 4) and PE (5, 6) are also auxotrophic for ethanolamine. The ethanolamine is transported into these cells and used to synthesize PE via the CDP-ethanolamine pathway. PE is subsequently methylated to form PC via the CDP-DAG pathway (Fig. 1).

The prevailing view has been that the CDP-choline pathway is a salvage pathway used by cells when the CDP-DAG pathway is compromised (11). However, recent studies have shown that the CDP-choline pathway contributes to PC synthesis even when wild-type cells are grown in the absence of exogenous choline (12). PC synthesized by the CDP-DAG pathway is constantly metabolized to choline and PA via the action of phospholipase D (13). The choline generated is then incorporated back into PC via the CDP-choline pathway and the PA is recycled back into PC, and other phospholipids (e.g. PI), via the CDP-DAG pathway (13). In fact, proper regulation of the CDP-choline pathway is important to overall lipid synthesis. For example, the activation of the CDP-choline pathway, due to the unregulated synthesis of CTP, results in significant increases in the synthesis of PC and PA and a decrease in the synthesis of PS (14). These changes are accompanied by an increase in total neutral lipid content at the expense of total phospholipids (14). The importance of the CDP-choline pathway to cell physiology is emphasized by the fact that the lethal phenotype of *sec14* mutants defective in the PI/PC transfer protein (Sec14p) is suppressed by mutations in the CDP-choline pathway (15, 16).

The role of the CDP-ethanolamine pathway in phospholipid metabolism and cell physiology has not been studied as extensively as the CDP-choline pathway. Although the genes encod...
Fig. 1. Pathways for the synthesis of the major phospholipids in S. cerevisiae. The pathways shown for the synthesis of phospholipids include the relevant steps discussed in the text. The CDP-ethanolamine, CDP-choline, and CDP-DAG pathways are indicated. The EKI1-encoded ethanolamine kinase and CKI1-encoded choline kinase reactions are indicated in the figure. A more comprehensive description of the additional steps in these pathways may be found in Refs. 1 and 2. The abbreviations used are: Etn, ethanolamine; P-Etn, phosphoethanolamine; CDP-Etn, CDP-ethanolamine; Cho, choline; P-Chol, phosphocholine; CDP-Chol, CDP-choline; PME, phosphatidyl ethanolamine; PDE, phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol; and CL, cardiolipin.

ing the enzymes catalyzing the last two steps in the CDP-ethanolamine pathway have been isolated and characterized (17, 18), little is known about the enzyme ethanolamine kinase (ATP:ethanolamine O-phosphotransferase, EC 2.7.1.82) that catalyzes the committed step in this pathway. In this paper we report the isolation and characterization of the EKI1 (ethanolamine kinase) gene encoding ethanolamine kinase in S. cerevisiae. Analysis of the EKI1 gene product expressed in Sf9 insect cells and the analysis of cells with deletions in the EKI1 and CKI1 genes indicated that the EKI1 gene product exhibited both ethanolamine kinase and choline kinase activities. The EKI1 gene product was primarily responsible for PE synthesis via the CDP-ethanolamine pathway, whereas the CKI1 gene product was primarily responsible for PC synthesis via the CDP-choline pathway. Unlike cki1 mutants, eki1Δ mutants did not suppress the essential function of Sec14p.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were reagent grade. Growth medium supplies were from Difco. Restriction endonucleases, modifying enzymes, and recombinant Vent DNA polymerase were from New England Biolabs. Polyclonal antibody and Silica Gel 60 thin layer chromatography plates were from EM Science.

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Methods

Strains, Plasmids, and Growth Conditions—The strains and plasmids used in this work are listed in Tables I and II, respectively. Methods for yeast growth and sporulation were performed as described previously (19, 20). Yeast cultures were grown in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or in complete synthetic medium minus inositol (2%) containing 2% glucose at 30 °C. The appropriate amino acid of complete synthetic medium was omitted for selection purposes. Escherichia coli strain DH5α was 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 cultures of DH5α carrying plasmids. Media were supplemented with either 2% (yeast) or 1.5% (E. coli) agar for growth on plates. Yeast cell numbers in liquid media were determined by microscopic examination with a hemacytometer or spectrophotometrically at an absorbance of 600 nm. The inositol excretion phenotype (22) of yeast strains was examined on complete synthetic medium (minus inositol) by use of growth of the inositol auxotrophic indicator strain MC13 (ino1) (21) as described by McGee et al. (23).

DNA Manipulations, Amplification of DNA by PCR, and DNA Sequencing—Plasmid and genomic DNA preparation, restriction enzyme digestion, and DNA ligations were performed by standard protocols (20). Conditions for the amplification of DNA by PCR were optimized as described by Innis and Gelfand (24). The annealing temperature for the PCR reactions was 55 °C, and extension times were typically between 2 and 3 min at 72 °C. PCR reactions were routinely run for a total of 30 cycles. DNA sequencing reactions were performed with the Prism Dye Deoxy Terminator Cycle sequencing kit and analyzed with an automated DNA Sequencer. Translations of yeast (25, 26) and E. coli (20) were performed as described previously. Plasmid maintenance and amplifications were performed in E. coli strain DH5α. Amplification of the plasmid pKSK1 was performed in E. coli strain Epicurian for experimentation.

Isolation of the EKI1 Gene—A DNA sequence encoding an open reading frame in the Saccharomyces Genome Data Base (locus YDR147W) (GenBank accession number Z50046) was named EKI1. A 2.9-kb DNA fragment containing 595 bp of the putative EKI1 promoter, its entire protein coding sequence, and 678 bp of the 3′-flanking sequence was obtained by PCR (primers 5′-TGTTTATCCTTTTCTCTACCGG-3′ and 5′-TGTTGTTTTGGTTGTTAATTATACGG-3′) using strain W303-1A genomic DNA as a template. The PCR product was ligated into the Srt1 site of the PCRScript™ AMP SK (+) cloning vector resulting in the creation of pKSK1. This plasmid was digested with PstI and SacI. The resulting 2.9-kb fragment containing the open reading frame and 595 bp of the promoter region and 678 bp of the 3′-flanking region was ligated into the PstI/SacI sites of Yep552 (27) to create plasmid pKSK3. This construct was then transformed into W303-1B and the indicated mutants for the overexpression of the EKI1 gene product.

A genomic copy of the EKI1 gene was also isolated. A search of the Saccharomyces Genome Data Base indicated that the EKI1 gene flanked the 3′-end of the KGD2 gene, which was isolated by Repetto and Thomas (28). We obtained the 5′-untranslated plasmid pG104/T1 that was used originally by Repetto and Tzagoloff (28) for isolation of the KGD2 gene. This plasmid contains an insert of yeast genomic DNA of approximately 8 kb. PCR and restriction enzyme analyses indicated that pG104/T1 contained the open reading frame and 5′- and 3′-flanking sequences. A 4.8-kb insert, containing the EKI1 gene with its promoter and 3′-untranslated region, was released from pG104/T1 by digestion with BamHI and SphI. This fragment was ligated into the HindIII/SphI sites of pUC18 to form plasmid pKSK4. Plasmid pKSK4 was used to construct a recombinant viral expression vector of the EKI1 gene in Sf9 insect cells.

Recombinant Viral Expression of the S. cerevisiae EKI1 Gene in Insect Cells—Plasmid pKSK4 was digested with BglII/BstI to release the genomic version of the yeast EKI1 gene. This DNA fragment was ligated into the BamHI/HincII sites of the pUC18 vector resulting in the formation of plasmid pKSK6. Plasmid pKSK6 was digested with BstYI/PstI to release the EKI1 open reading frame, which was then ligated into the BamHI/PstI sites of the baculovirus vector pVL1393 to form plasmid pKSK7.

Sf9 cells were maintained and grown as monolayers in TMNFH medium containing containing 10% heat-inactivated fetal bovine serum (medium A) (29). Sf9 cells were co-transfected with pKSK7 and BaculoloGold™ Autographa californica DNA (PharMingen) using the CaCl2 method. Sf9 infection procedures followed the methods described by O’Reilly et al. (29). For EKI1 expression, Sf9 cells (1 × 106 cells grown in 75-cm2 tissue culture flasks) were infected with a viral multiplicity of 10 and grown for 48 h in medium A. The infected cells were collected by gentle trituration with medium, harvested by centrifugation, and washed twice with phosphate-buffered saline. The final cell pellet was snap-frozen over dry ice and stored at −80 °C.

Construction of eki1Δ and cki1Δ Mutants—The plasmid pKSK1 was digested with BglII/BamHI to remove the approximately 70% EKI1 coding region. A 1.8-kb TRP1 disruption cassette, derived from plasmid pA523 (30) by BglII/SnaI digestion, was inserted into the BglII/BamHI
temperature-sensitive sec14 phan. Disruption of the chromosomal copy of the one-step gene disruption technique (31). Transformants were selected for the disruption cassette was released from the plasmid pKSK2 by digestion with SacI that grew on medium without tryptophan. One of the eki1 transformants that grew on medium without histidine and without tryptophan and histidine, respectively. One of the transformed colonies that grew on medium without histidine and with-OP1 MATa, opi1–1, lys2 22 CKI1 double mutants. A linear 4-kb CKI1 disruption was genomic DNA isolated from transformed colonies that grew on medium without histidine and with-CKI1 gene was disrupted in the mutant strain (strain CTY5-2D) (31). The appro-
appropriate transformants were isolated, and the disruptions of the chromosomal copy that grew on medium without tryptophan. One of the CKI1 gene of pCTY301 (chil-284:His3) 15 HILysisI site of the CKI1 gene of pCTY301 (chil-284:His3) 15 2-µm plasmid containing a genomic copy of EKI1 28 New England Biolabs pKS4 BamH1/SphiI fragment of the EKI1 gene from pG104/T1 ligated into the BamH1/SphiI sites of pUC18 This work pKL393 Baculovirus expression vector Invitrogen pKSK7 EKI1 gene from pKSK4 ligated into the BamH1/PstI sites of pVL1393 This work

Preparation of Cell Extracts—All steps were performed at 5 °C. Yeast cells were disrupted with glass beads with a Mini-Bead-Beater (Biospec Products) in 50 mM Tris–HCl buffer (pH 7.5) containing 1 mM Na3EDTA, 0.3 M sucrose, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethanesulfonyl fluoride, 1 mM benzamide, 5 µg/ml aprotinin, 5 µg/ml leupeptin, and 5 µg/ml pepstatin (33). Glass beads and cell debris were removed by centrifugation at 1,500 × g for 5 min. The supernatant was used as the cell extract. Insect cells were disrupted by sonic oscillation in 50 mM Tris–HCl buffer (pH 7.5) containing 0.3 M sucrose, 1 mM Na3EDTA, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethanesulfonyl fluoride, 1 mM benzamide, 5 µg/ml aprotinin, 5 µg/ml leupeptin, and 5 µg/ml pepstatin (34). The disrupted cell suspension was centrifuged at 1,500 × g for 5 min to remove unbroken cells and cell debris. The supernatant was used as the cell extract.

Enzyme Assays, Protein Determination, and Analysis of Kinetic Data—Ethanolamine kinase activity was measured for 20 min at 30 °C by following the phosphorylation of [1,2-14C]ethanolamine (5,000 cpm/mmol) with ATP. The reaction mixture contained 50 mM Tris–HCl buffer (pH 8.5), 5 mM ethanolamine, 10 mM ATP, 10 mM MgSO4, and enzyme protein in a final volume of 50 µl. The product phosphoethanolamine was identified by thin layer chromatography on silica gel plates using the solvent system methanol, 0.6% sodium chloride, ammonium hydroxide (10:10:1) (35). The position of the labeled phosphoethanolamine on chromatograms was determined by fluorography using EN3HANCE and compared with standard phosphoethanolamine. The amount of labeled product was determined by scintillation counting. All assays for the activity were performed in triplicate with the triplicates representing the mean ± SD.

Site-directed mutagenesis strategy used was to construct chi1A and eki1A chi1A double mutants. A linear 4-kb CKI1 disruption cassette was released from the plasmid pCTY307 (15) by digestion with ClaI/HpaI. Strain W303-1A was transformed with this DNA fragment to disrupt the chromosomal copy of the CKI1 gene in strain CTY5-2D (31). One of the chi1A mutants that we isolated was designated strain KS101. A similar strategy was used to construct CKI1 chi1A double mutants. A linear 4-kb CKI1 disruption cassette was released from the plasmid pCTY307 (15) by digestion with ClaI/HpaI. Strain W303-1A was transformed with this DNA fragment to disrupt the chromosomal copy of the CKI1 gene (31). Transformants were selected for their ability to grow on complete synthetic medium without histidine. Similarly, the CKI1 gene was disrupted in the eki1A mutant strain KS101, and transformants were selected for their ability to grow without tryptophan and histidine. Disruption of the chromosomal copy of the CKI1 gene in these cells was confirmed by PCR (primers, 5′-TTGG-CATTATCTGAAAGGGG-3′ and 5′-GGAAGTCAATGATGTAGACG-3′) with the extension time increased to 2.5 min. The template for the PCRs used to confirm the CKI1 disruption was genomic DNA isolated from transformed colonies that grew on medium without histidine and without tryptophan and histidine, respectively. One of the chi1A mutants and one of the eki1A chi1A double mutants that we isolated were designated strains KS105 and KS106, respectively.

The EKI1 and CKI1 deletion cassettes were used to transform a temperature-sensitive sec14 mutant (strain CTY5-2D) (31). The appropriate transformants were isolated, and the disruptions of the chromosomal copies of the EKI1 and CKI1 genes in the sec14A background were confirmed by PCR as described above. The eki1A sec14A and chi1A sec14A double mutants were designated KS118 and KS119, respectively.

| Strain     | Relevant characteristics | Source or Ref. |
|------------|--------------------------|---------------|
| E. coli    | F −, 800lacZΔM15, 8lacZYA-argF169, deoR, recA1, endA1, hsdR17(rK-m−), phoA, supE44, λ thi-1, gyrA96, relA1 | Stratagene |
| Epicurian  | Δ(mcrA1833/mcrB-hsdSMR-mrr173, endA1, supE44, thi-1, recA1, gyrA96, relA1 | Stratagene |
| S. cerevisiae Coli' XL-1 | Δ(mcrA1833/mcrB-hsdSMR-mrr173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac[P proAB, lacP2Δ, M15, Th1(Kan') | Stratagene |

### Table I

| Strains used in this work | Relevant characteristics |
|--------------------------|--------------------------|
| **Strain**               | **Source or Ref.**       |
| W303–1A                  | This work                |
| W303–1B                  | This work                |
| KS101                    | This work                |
| KS105                    | This work                |
| KS106                    | This work                |
| MC13                     | This work                |
| OP1                      | This work                |
| CTYS-2D                  | This work                |
| KS118                    | This work                |
| KS119                    | This work                |

### Table II

| Plasmids used in this work | Relevant characteristics | Source or Ref. |
|---------------------------|--------------------------|---------------|
| pCRScript™               | Cloning vector derived from the pBluescript® II SK (+) | Stratagene |
| AMP                      | phagemid, modified SpfI restriction endonuclease | This work |
| SK (+)                   | target sequence          | This work |
| pKSK1                    | EKI1 gene derived from PCR ligated into the SpfI site of pCRScript™ AMP SK (+) | This work |
| pJS52                    | Plasmid containing a TRP1 disruption cassette | This work |
| pKSK2                    | TRP1 disruption cassette from pJS52 ligated into the BglII/BsoI sites of plasmid pKSK1 | This work |
| YEp352                   | Multicopy E. coli yeast shuttle vector containing URA3 | This work |
| pKSK3                    | EKI1 gene (PstI/Sacl fragment) from pKSK1 ligated into the PstI/Sacl sites of YEp352 | This work |
| pCTY307                  | H1S3 disruption cassette ligated into the BamH1 site of the CKI1 gene of pCTY301 (chil-284:His3) | This work |
| pG104/T1                 | 2-µm plasmid containing a genomic copy of EKI1 | New England Biolabs |
| pUC18                    | Multicopy E. coli cloning vector containing Amp' | This work |
| pKSK4                    | BamH1/SphiI fragment of the EKI1 gene from pG104/T1 ligated into the BamH1/SphiI sites of pUC18 | This work |
| pVL1393                  | Baculovirus expression vector | Invitrogen |
| pKSK7                    | EKI1 gene from pKSK4 ligated into the BamH1/PstI sites of pVL1393 | This work |
isolation of the S. cerevisiae EKI1 Gene Encoding Ethanolamine Kinase and the Deduced Primary Structure of Its Encoded Protein—The EKI1 gene was identified in the Saccharomyces Genome Data Base on the basis that its deduced amino acid sequence showed 40% sequence identity to the C-terminal amino acid sequence of the CK11-encoded choline kinase of S. cerevisiae (45, 46) (Fig. 2). The homologous region of these proteins contain a phosphotransferase consensus sequence domain (47), which is also present in the ethanolamine kinase sequence of the Drosophila melanogaster gene for ethanolamine kinase (47). The de novo cloned amino acid sequence encoded by the S. cerevisiae EKI1 and CK11 genes and the easily shocked gene of D. melanogaster were determined by liquid scintillation counting.

The amino acid sequence of the EKI1 gene from D. melanogaster (47) showed 40% sequence identity to the C-terminal amino acid sequence of the S. cerevisiae CK11-encoded choline kinase (45, 46) (Fig. 2). The homologous region of these proteins contain a phosphotransferase consensus sequence domain (47), which is also present in the ethanolamine kinase sequence of the Drosophila melanogaster gene for ethanolamine kinase (47). The de novo cloned amino acid sequence encoded by the S. cerevisiae EKI1 and CK11 genes and the easily shocked gene of D. melanogaster were determined by liquid scintillation counting.

**RESULTS**

**Isolation of the S. cerevisiae EKI1 Gene Encoding Ethanolamine Kinase and the Deduced Primary Structure of Its Encoded Protein—**The EKI1 gene was identified in the Saccharomyces Genome Data Base on the basis that its deduced amino acid sequence showed 40% sequence identity to the C-terminal amino acid sequence of the CK11-encoded choline kinase of S. cerevisiae (45, 46) (Fig. 2). The homologous region of these proteins contain a phosphotransferase consensus sequence domain (47), which is also present in the ethanolamine kinase encoded by the easily shocked (eas) gene of Drosophila melanogaster.
EKI1 Gene Encoding Ethanolamine Kinase from S. cerevisiae

Cell extracts were prepared from Sf-9 insect cells and assayed for ethanolamine kinase and choline kinase activities as described under “Experimental Procedures.” The specific activities of ethanolamine kinase and choline kinase in the control Sf-9 insect cells were 0.002 and 0.003 units/mg, respectively. The break in the activity axis is between 8 and 100 units/mg. The values reported were the average of three separate experiments ± S.D. WT, wild-type.

Fig. 3. Ethanolamine kinase and choline kinase activities in S. cerevisiae wild-type cells and in Sf-9 insect cells overexpressing the EKI1 gene. Cell extracts were prepared from the indicated S. cerevisiae and Sf-9 insect cells and assayed for ethanolamine kinase and choline kinase activities as described under “Experimental Procedures.” The specific activities of ethanolamine kinase and choline kinase in the control Sf-9 insect cells were 0.002 and 0.003 units/mg, respectively. The break in the activity axis is between 8 and 100 units/mg. The values reported were the average of three separate experiments ± S.D. WT, wild-type.

**Table III**

Kinetic constants for the EKI1-encoded kinase

| Substrate | Vmax(app) (nmol/min/mg) | Km(app) (mM) | Vmax(app)/Km(app) (app)
|-----------|-------------------------|-------------|------------------------|
| Ethanolamine | 346                     | 0.171       | 2023                   |
| Choline    | 249                     | 0.275       | 906                    |

Cell extracts were prepared from Sf-9 insect cells and assayed for ethanolamine kinase and choline kinase activities as a function of the concentration (0–4 mM) of ethanolamine or choline as described under “Experimental Procedures.” The concentration of ATP in the enzyme assays was maintained at 10 mM. The data were analyzed according to the Michaelis-Menten equation using the EZ-FIT Enzyme Model Fitting Program.

not rule out the possibility that the EKI1 gene was a regulatory gene that controlled the expression of ethanolamine kinase activity in S. cerevisiae. To test further the hypothesis that the EKI1 gene was the structural gene encoding ethanolamine kinase, we used heterologous expression of the gene in Sf-9 insect cells. The EKI1 gene was placed within the genome of baculovirus under control of the polyhedrin promoter and expressed by viral infection of Sf-9 cells. Infection of the cells with the baculovirus containing the EKI1 gene resulted in the massive overexpression (165,500-fold) of ethanolamine kinase activity when compared with uninfected cells (Fig. 3). This massive level of ethanolamine kinase expression was equivalent to a 675-fold purification over the activity expressed in the cell extract of wild-type S. cerevisiae (Fig. 3).

We also examined the ability of the EKI1 gene product to utilize choline as a substrate. Choline was used for this analysis because of the homology that the EKI1 gene product showed to the CKI1-encoded choline kinase (Fig. 2). The choline kinase activity in wild-type cells was 11-fold higher than the ethanolamine kinase activity in wild-type cells. There was only a 1.1-fold increase in choline kinase activity in wild-type cells bearing the EKI1 gene on the multicopy plasmid when compared with the control cells (Fig. 3). Infection of the Sf-9 insect cells with the baculovirus containing the EKI1 gene resulted in the massive overexpression (76,000-fold) of choline kinase activity (Fig. 3). The choline kinase activity expressed in the infected insect cells was 41-fold greater than the choline kinase activity expressed in wild-type S. cerevisiae. The ethanolamine kinase activity was 1.5-fold greater than the choline kinase activity in the insect cells infected with virus containing the yeast EKI1 gene. These data provided strong evidence that the EKI1 gene encoded an enzyme with both ethanolamine kinase and choline kinase activities.

The dependence of the EKI1-encoded kinase on the concentrations of ethanolamine and choline was examined using the cell extract of Sf-9 insect cells expressing the EKI1 gene. The kinase exhibited saturation kinetics with respect to ethanolamine and to choline using a saturating concentration (10 mM) of ATP. Ethanolamine was the preferred substrate for the enzyme based on the relative values for Vmax and K▼, (Table III). The specificity constant (Vmax/K▼) for ethanolamine was 2.2-fold higher than that for choline.

**Deletion of the EKI1 Gene**—The EKI1 gene was deleted to test further the hypothesis that its gene product encoded an ethanolamine/choline kinase enzyme. In addition, the availability of an eki1Δ mutant would allow us to examine whether the EKI1 gene was essential for cell growth and to examine the role this gene plays in phospholipid metabolism. A genomic construct containing the EKI1 gene was manipulated to delete about 70% of its coding sequence. The EKI1 deletion construct was introduced into the genome of haploid cells by homologous recombination as described under “Experimental Procedures.”

Information available on-line at the following address: http://psort.nibb.ac.jp/form.html.

Information available on-line at the following address: http://www.genome.ad.jp/sit/motif.html.
Haploid eki1Δ mutant cells were viable and exhibited growth properties similar to wild-type control cells when grown vegetatively in YEPD medium at 30 °C. However, the eki1Δ mutant grew at a slightly slower rate in complete synthetic medium at 30 °C when compared with the control cells. Microscopic examination of eki1Δ mutant cells showed no apparent gross morphological differences when compared with wild-type cells when grown in either complete synthetic medium or YEPD medium. In addition, mating and sporulation were not affected in the eki1Δ mutant. Overall, these results indicated that the EKI1 gene was not essential for cell growth under typical laboratory growth conditions.

The EKI1 gene was also deleted in a cki1Δ mutant background to construct an eki1Δ cki1Δ double mutant. Haploid eki1Δ cki1Δ mutant cells were viable and appeared normal by microscopic examination. Like the eki1Δ mutant, the eki1Δ cki1Δ double mutant grew slightly slower than the control cells in complete synthetic medium. The cki1Δ mutant exhibited growth properties similar to wild-type control cells when grown vegetatively in YEPD medium and in complete synthetic medium at 30 °C.

The eki1Δ mutant and the eki1Δ cki1Δ double mutant were examined for an inositol excretion phenotype (22). This phenotype is the result of the derepression of the INO1 gene (51) and is a characteristic trait of mutants defective in the structural genes for several phospholipid biosynthetic enzymes (50, 51). Growth of the ino1 mutant was used as an indicator of the phenotype, and the opi1 mutant, which excretes inositol (22) due to unregulated derepression of the INO1 gene (50, 51), was used as a positive control. Neither one of these mutants exhibited the inositol excretion phenotype. As described previously (12, 52), the cki1Δ mutant did not exhibit the inositol excretion phenotype.

Sec14p is a PI/PC transfer protein that is essential for cell viability and vesicle budding from the Golgi complex (53, 54). It has been proposed that a function of Sec14p is to down-regulate the synthesis of PC via the CDP-choline pathway (53). It appears that too much PC synthesized via the CDP-choline pathway is detrimental to the secretory process (55). Mutations in the Sec14p gene can suppress (i.e. bypass) the lethal phenotype of a sec14 mutant (15, 16). For example, a cki1 sec14Δ double mutant is viable at the restrictive temperature (15). Thus, a block in the CDP-choline pathway removes the need for Sec14p function (15, 53). Given the fact that the EKI1 gene product exhibited ethanolamine kinase and choline kinase activities, we examined whether the eki1Δ mutation would suppress the essential function of Sec14p. An eki1Δ sec14Δ double mutant was constructed as described under “Experimental Procedures.” We also constructed a cki1Δ sec14Δ double mutant in the same genetic background to be used as a positive control. The sec14Δ mutant, the eki1Δ sec14Δ double mutant, and the cki1Δ sec14Δ double mutant were grown at 25 °C (permissive temperature) and 37 °C (restrictive temperature). As described previously (15), the cki1Δ sec14Δ double mutant grew at the restrictive temperature. On the other hand, the eki1Δ sec14Δ double mutant was not viable at the restrictive temperature, whereas the mutant grew normally at the permissive temperature. Thus, the mutation in the EKI1 gene did not suppress the essential Sec14p function.

Ethanolamine Kinase and Choline Kinase Activities in the S. cerevisiae eki1Δ Mutant, cki1Δ Mutant, and eki1Δ cki1Δ Double Mutant—The eki1Δ mutant was grown to exponential phase, and cell extracts were prepared and assayed for ethanolamine kinase and choline kinase activities. Ethanolamine kinase activity was reduced by 40% in the eki1Δ mutant when compared with the ethanolamine kinase activity found in the wild-type parent strain (Fig. 4). However, the expression of choline kinase activity was not affected in the eki1Δ mutant (Fig. 4).

Transformation of the eki1Δ mutant with the multicopy plasmid containing the EKI1 gene resulted in an overexpression of ethanolamine kinase and choline kinase activities of 8- and 1.3-fold, respectively, when compared with these activities in the eki1Δ mutant (Fig. 4).

To examine the expression of the ethanolamine kinase and choline kinase activities encoded by the EKI1 gene product in the absence of the CKII-encoded choline kinase, we utilized a cki1Δ mutant. Ethanolamine kinase and choline kinase activities were reduced in the cki1Δ mutant by 82 and 98%, respectively, when compared with the wild-type parent (Fig. 4). These data were consistent with previous studies showing that the CKI1 gene product possessed both choline kinase and ethanolamine kinase activities (45, 56). Transformation of the cki1Δ mutant with the EKI1 gene on the multicopy plasmid resulted in increases in ethanolamine kinase and choline kinase activities of 16- and 2.8-fold, respectively, when compared with these activities in the cki1Δ mutant (Fig. 4).

The eki1Δ cki1Δ double mutant was examined for ethanolamine kinase and choline kinase activities. These activities were not detectable in the double mutant (Fig. 4). Thus, the EKI1 and CKI1 genes accounted for all of the measurable ethanolamine kinase and choline kinase activities in S. cerevisiae. We expressed the EKI1 gene in the eki1Δ cki1Δ double mutant to explore further the ethanolamine kinase and choline kinase activities encoded by the EKI1 gene. In this double mutant background, the ethanolamine kinase activity (2.6 units/mg) encoded by the EKI1 gene was 3.6-fold greater than the choline kinase activity (0.72 units/mg) encoded by the gene (Fig. 4). These data indicated that ethanolamine was preferred over choline as a substrate and were also consistent with the data using the enzyme derived from insect cells expressing the EKI1 gene.

**Effect of the eki1Δ, cki1Δ, and eki1Δ cki1Δ Mutations on the Composition of the CDP-Ethanolamine Pathway Intermediates.—**The CDP-ethanolamine pathway intermediates include ethanolamine, phosphoethanolamine, and CDP-ethanolamine (Fig. 1). Cells were labeled with [1,2-14C]ethanolamine to steady state to analyze the composition of the CDP-ethanolamine pathway intermediates. The eki1Δ and the cki1Δ mutants exhibited alterations in the incorporation of ethanolamine into the total pool of CDP-ethanolamine pathway intermediates (Fig. 5A). The deletion of the EKI1 gene resulted in a 1.7-fold increase in the amount of ethanolamine incorporated into this pool when compared with the wild-type control. The deletion of the CKI1 gene resulted in a 70% decrease in the ethanolamine incorporated into the pool of CDP-ethanolamine pathway intermediates. The incorporation of ethanolamine into the inter-
mediates of the eki1Δ cki1Δ double mutant was similar to that of the cki1Δ mutant. The effects of the eki1Δ and cki1Δ mutations on the relative amounts of the CDP-ethanolamine pathway intermediates are shown in Fig. 5B. In the control cells, 8.7 and 4% of the label was incorporated into phosphoethanolamine and CDP-ethanolamine, respectively, whereas most of the label was found in ethanolamine. The deletion of the EKI1 gene resulted in dramatic decreases in the relative amounts of phosphoethanolamine (77%) and CDP-ethanolamine (85%), respectively. The deletion of the CKI1 gene resulted in a small decrease in the amount of phosphoethanolamine (12%) and a small increase in the amount of CDP-ethanolamine (1.5-fold). The only intermediate found in the eki1Δ cki1Δ double mutant was ethanolamine. Thus, the CDP-ethanolamine pathway was totally blocked in the double mutant.

**Effect of the eki1Δ, cki1Δ, and eki1Δ cki1Δ Mutations on the Composition of the CDP-Choline Pathway Intermediates—**

Cells were labeled with [methyl-3H]choline to steady state to analyze the composition of the CDP-choline pathway intermediates, which include choline, phosphocholine, and CDP-choline (Fig. 1). Data for the incorporation of choline into the total pool of CDP-choline pathway intermediates is shown in Fig. 6A. The incorporation of choline into the CDP-choline pathway intermediates was about 40-fold greater than the incorporation of ethanolamine into the CDP-ethanolamine pathway intermediates (note the difference in the y axes labels between Figs. 5A and 6A). Furthermore, the choline that was transported into the cells was more readily incorporated into the CDP-choline pathway intermediates when compared with the incorporation of ethanolamine into the CDP-ethanolamine pathway intermediates (Figs. 5B and 6B). The deletion of the EKI1 gene did not have a significant effect on the incorporation of choline into the CDP-choline pathway intermediates, whereas the deletion of the CKI1 gene resulted in a 90% decrease in the incorporation of choline into the intermediates. The amount of choline incorporated into the pool of intermediates in the eki1Δ cki1Δ double mutant was similar to that of the cki1Δ mutant. The effects of the eki1Δ and cki1Δ mutations on the relative amounts of the CDP-choline pathway intermediates are shown in Fig. 6B. The deletion of the EKI1 gene resulted in a 2.4-fold increase in the amount of choline and a 50% decrease in the amount of phosphocholine when compared with the wild-type control cells. Deletion of the CKI1 had a more dramatic effect on the relative amounts of choline and phosphocholine when compared with the deletion of the EKI1 gene. The amount of choline increased 4.3-fold and the amount of phosphocholine decreased by 89% when compared with the control cells. The relative amounts of CDP-choline were not significantly affected in the eki1Δ and cki1Δ mutants. Phosphocholine and CDP-choline were not detected in the eki1Δ cki1Δ double mutant. Thus, the CDP-choline pathway was totally blocked in the double mutant.

**Effect of the eki1Δ, cki1Δ, and eki1Δ cki1Δ Mutations on Phospholipid Composition—**
The phospholipid composition was analyzed in the eki1Δ mutant, the cki1Δ mutant, and the eki1Δ cki1Δ double mutant. Cells were grown in complete synthetic medium without inositol, ethanolamine, and choline to remove the regulatory effects these precursors have on phospholipid synthesis (1, 50). The composition of phospholipids was examined by labeling cells to steady state with [32P]Pi, [1,2-14C]ethanolamine, and [methyl-3H]choline. [32P]Pi will be incorporated into phospholipids synthesized by the CDP-ethanolamine, CDP-choline, and CDP-DAG pathways. The labeled ethanolamine will only be incorporated into PE via the CDP-ethanolamine pathway, whereas the labeled choline will only be incorporated into PC synthesized by the CDP-choline pathway (1, 50). The effects of the eki1Δ and cki1Δ mutations on phospholipid composition are shown in Fig. 7A.
These mutations did not have a significant effect on the incorporation of $^{32}$P into phospholipids and did not have a major effect on the overall phospholipid composition when compared with the wild-type control cells. The eki1Δ chi1Δ double mutant showed a 4% decrease in the amount of PI, the eki1Δ mutant and the eki1Δ chi1Δ double mutant showed a 5% increase in the amount of PE, and the chi1Δ mutant showed a 6% decrease in the amount of PC.

Radiolabeled ethanolamine was incorporated into PE and PC during the labeling experiments (Fig. 7B). This indicated that PE was synthesized by the CDP-ethanolamine pathway in the eki1Δ and chi1Δ mutants as well as in the control cells. The fact that the label was also incorporated into PC indicated that the PE synthesized was used for the synthesis of PC via the phospholipid methyltransferase enzymes that are used in the CDP-DAG pathway (1, 50). The ethanolamine label was not incorporated into PE and PC in the eki1Δ chi1Δ double mutant. This result was consistent with the labeling experiments of the CDP-ethanolamine pathway intermediates where the label was only found in ethanolamine (Fig. 5B). The data shown in Fig. 7B are plotted as the ratio of the cpm of $^{14}$C incorporated into PE + PC to the cpm of $^{32}$P incorporated into these phospholipids. This allowed us to determine the effects of the eki1Δ and chi1Δ mutations on the pathways by which cells synthesized PE. The eki1Δ mutant showed an 80% decrease in the ratio of PE + PC when compared with the control cells. These results indicated that the deletion of the EKI1 gene resulted in a major decrease in the utilization of the CDP-ethanolamine pathway for PE synthesis. The deletion of the EKI1 gene did not have a significant effect on the synthesis of PE via the CDP-ethanolamine pathway (Fig. 7B).

Radiolabeled choline was incorporated into PC in the eki1Δ and chi1Δ mutants during the labeling experiments (Fig. 7C). This indicated that PC was synthesized by the CDP-choline pathway in both mutants. The data shown in Fig. 7C are plotted as the ratio of the cpm of $^{3}$H incorporated into PC to the cpm of $^{32}$P incorporated into PC. This allowed us to determine the effects of the eki1Δ and chi1Δ mutations on the pathways by which cells synthesized PC (40). The deletion of the EKI1 gene did not have a significant effect on the ratio of $^{3}$H/$^{32}$P when compared with the control cells. However, the deletion of the CKI1 gene resulted in an 91% decrease in this ratio (Fig. 7C). These results indicated that the deletion of the CKI1 gene resulted in a major decrease in the utilization of the CDP-choline pathway for PC synthesis. Labeled choline was not incorporated into PC in the eki1Δ chi1Δ double mutant. This was consistent with the labeling of the CDP-choline pathway intermediates where the label was only found in choline (Fig. 6B).

**DISCUSSION**

There have been a number of conflicting reports as to whether a single enzyme catalyzes the phosphorylation of ethanolamine and choline in various systems (46, 57). Results of genetic and biochemical experiments have indicated that nearly all of the choline kinase activity in *S. cerevisiae* is encoded by the CKI1 gene (45, 56). The purified CKI1-encoded choline kinase enzyme also catalyzes the phosphorylation of ethanolamine, albeit with only 14% of the activity when choline is used as the substrate (50). Although choline kinase activity is barely detectable in a chi1Δ mutant, cell extracts derived from the mutant exhibit ethanolamine kinase activity (45). These data suggest that a distinct ethanolamine kinase enzyme exists in *S. cerevisiae* (46). Indeed, such an enzyme exists, and in this work we isolated the structural gene that encodes it.

The EKI1 gene was identified on the basis upon a deduced amino acid sequence that showed homology to the CKI1-encoded choline kinase (46). The EKI1 gene, which is found on chromosome IV, was isolated and characterized. A multicopy plasmid containing the EKI1 gene directed the overexpression of ethanolamine kinase activity in *S. cerevisiae* wild-type cells, in an eki1Δ mutant, in a chi1Δ mutant, and in an eki1Δ chi1Δ double mutant. Moreover, the heterologous expression of the EKI1 gene in SF-9 insect cells resulted in a massive overexpression of ethanolamine kinase activity. The deletion of the EKI1 gene in *S. cerevisiae* resulted in a 40% reduction in ethanolamine...
mine kinase activity. The remaining ethanolamine kinase activity in the eki1Δ mutant was attributed to the ethanolamine kinase activity of the CKI1-encoded choline kinase. Collectively, these data provided conclusive evidence for the identification of the EKI1 gene as the structural gene encoding ethanolamine kinase.

The EKI1 gene product also exhibited choline kinase activity. Based on the specificity constants for ethanolamine and choline, and the levels of overexpression of the EKI1 gene product in the eki1Δ mutant, cki1Δ mutant, and the eki1Δ cki1Δ double mutant, ethanolamine was the preferred substrate for the enzyme. These analyses also indicated that together the EKI1 and CKI1 gene products accounted for all of the ethanolamine kinase and choline kinase activities in S. cerevisiae.

The eki1Δ mutant, cki1Δ mutant, and eki1Δ cki1Δ double mutant were used to examine the contributions of the EKI1 and CKI1 gene products to phospholipid synthesis via the CDP-ethanolamine and CDP-choline pathways. The results of these studies showed that the synthesis of phospholipids via the CDP-ethanolamine pathway was dramatically reduced in the eki1Δ mutant, whereas the synthesis of phospholipids via the CDP-choline pathway was dramatically reduced in the cki1Δ mutant. These results also showed that the EKI1 gene product made a small contribution to phospholipid synthesis via the CDP-choline pathway and that the CKI1 gene product made a small contribution to phospholipid synthesis via the CDP-ethanolamine pathway. Thus, the EKI1 and CKI1 gene products exhibited overlapping functions with respect to phospholipid synthesis. These results agreed with previous data showing that in the absence of a functional CDP-choline pathway, ethanolamine kinase activity was the major enzyme responsible for phospholipid synthesis. Whether the CDP-DAG pathway enzymes were synthesized an essentially normal complement of membrane phospholipids. Whether the CDP-DAG pathway enzymes were regulated in the eki1Δ mutant and cki1Δ mutant backgrounds to compensate for these mutations will require additional studies.

The deletion of the EKI1 gene in S. cerevisiae revealed that the EKI1 gene was not essential for growth under typical laboratory conditions. The eki1Δ mutant did not exhibit a significant growth phenotype. The yeast eki1Δ mutant also lacks a growth phenotype (45). However, the cki1Δ mutant exhibits a strong choline excretion phenotype in a sec14ts growth phenotype (45). However, the cki1Δ mutant also lacks a growth phenotype (45). This phenotype is dependent on the SPO14/PLD1-encoded phospholipase D-mediated turnover of PC, synthesized by the CDP-DAG pathway, and the inability of cells to reincorporate the choline back into PC by the CDP-choline pathway (12, 13). As discussed above, the cki1Δ mutation can suppress the sec14ts phenotype (15). However, the cki1Δ mutation does not suppress the sec14ts phenotype if the SPO14 gene is also deleted (13). This situation holds for other CDP-choline pathway mutations (i.e. cct1 and cpl1) (12, 13), which can also suppress the sec14ts phenotype (15). Thus, the CDP-choline pathway and phospholipase D are both important for Sec14p-mediated secretion and viability in S. cerevisiae (55). Although the EKI1-encoded ethanolamine kinase possesses choline kinase activity, the deletion of the EKI1 gene did not suppress the sec14ts phenotype. The EPT1-encoded ethanolamine phosphotransferase, which catalyzes the final step in the CDP-ethanolamine pathway (Fig. 1), possesses choline phosphotransferase activity (18). Like the cki1Δ mutation, the cpl1 mutation does not suppress the sec14ts phenotype (15). Thus, the CDP-choline and CDP-ethanolamine pathways do not appear to have overlapping functions with respect to Sec14p function.

Utilization of the CDP-ethanolamine and CDP-choline pathways by S. cerevisiae requires the transport of ethanolamine and choline, respectively, into the cell (58). Ethanolamine and choline are both transported by a single transporter encoded by the CTR gene (58, 59). The in vivo labeling experiments showed that the incorporation of both ethanolamine and choline into cells was very reduced in the cki1Δ mutant and the eki1Δ cki1Δ double mutant when compared with wild-type cells. On the other hand, the incorporation of ethanolamine and choline into the eki1Δ mutant was actually greater than that of wild-type cells. These results suggested that the deletion of the EKI1 and CKI1 genes, and/or the defect in PE and PC synthesis via the CDP-ethanolamine and CDP-choline pathways, affected either the expression and/or function of the CTR-encoded transporter. These studies also showed that the incorporation of choline into wild-type and mutant cells was much greater than that of ethanolamine. Moreover, the utilization of choline for phospholipid synthesis via the CDP-choline pathway was greater than that of ethanolamine for phospholipid synthesis via the CDP-ethanolamine pathway. The availability of the mutants described in this study will permit additional studies to address the regulation of the choline/ethanolamine transporter as well as the utilization of choline and ethanolamine for phospholipid synthesis.

The EKI1-encoded ethanolamine kinase is a novel enzyme not described previously. It differed from the ethanolamine kinase encoded by the eas gene of D. melanogaster. The ethanolamine kinase from Drosophila is highly specific for ethanolamine (48), which is in sharp contrast with the yeast EKI1-encoded ethanolamine kinase which also utilized choline as a substrate. Pavlidis et al. (48) have shown that a mutation in the eas gene causes seizure, neuronal failure, and paralysis. These phenotypes have been attributed to a defect in the synthesis of PE via the CDP-ethanolamine pathway (48). Further detailed insights into the physiological role of the EKI1-encoded ethanolamine kinase and the CDP-ethanolamine pathway are likely to be revealed through molecular genetic approaches with the yeast system.

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