Phosphorylation of the Yeast Choline Kinase by Protein Kinase C:
IDENTIFICATION OF SER\textsuperscript{25} AND SER\textsuperscript{30} AS MAJOR SITES OF PHOSPHORYLATION*

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Abstract

The Saccharomyces cerevisiae CKI\textsubscript{1}-encoded choline kinase catalyzes the committed step in phosphatidylcholine synthesis via the Kennedy pathway. The enzyme is phosphorylated on multiple serine residues, and some of this phosphorylation is mediated by protein kinase A. In this work, we examined the hypothesis that choline kinase is also phosphorylated by protein kinase C. Using choline kinase as a substrate, protein kinase C activity was dose- and time-dependent, and dependent on the concentrations of choline kinase ($K_m = 27 \mu g/ml$) and ATP ($K_m = 15 \mu M$). This phosphorylation, which occurred on a serine residue, was accompanied by a 1.6-fold stimulation of choline kinase activity. The synthetic peptide SRSS\textsuperscript{25}QRRHS ($V_{max}/K_m = 17.5 \text{ mM}^{-1} \text{ mmol min}^{-1} \text{ mg}^{-1}$) that contains the protein kinase C motif for Ser\textsuperscript{25} was a substrate for protein kinase C. A Ser\textsuperscript{25} to Ala (S25A) mutation in choline kinase resulted in a 60% decrease in protein kinase C phosphorylation of the enzyme. Phosphopeptide mapping analysis of the S25A mutant enzyme confirmed that Ser\textsuperscript{25} was a protein kinase C target site. In vivo, the S25A mutation correlated with a decrease (55%) in phosphatidylcholine synthesis via the Kennedy pathway whereas an S25D phosphorylation site mimic correlated with an increase (44%) in phosphatidylcholine synthesis. Whereas the S25A (protein kinase C site) mutation did not affect the phosphorylation of choline kinase by protein kinase A, the S30A (protein kinase A site) mutation caused a 46% reduction in enzyme phosphorylation by protein kinase C. A choline kinase synthetic peptide (SQRRHS\textsuperscript{30}LTRQ) containing Ser\textsuperscript{30} was a substrate ($V_{max}/K_m = 3.0 \text{ mM}^{-1} \text{ mmol min}^{-1} \text{ mg}^{-1}$) for protein kinase C. Comparison of phosphopeptide maps of the wild type and S30A mutant choline kinase enzymes phosphorylated by protein kinase C confirmed that Ser\textsuperscript{30} was also a target site for protein kinase C.

PC\textsuperscript{1} is the major phospholipid in the membranes of eukaryotic cells (1–4). It is a structural component of cell membranes, and a source of lipid molecules (e.g., lysophosphatidylcholine, diacylglycerol, lysophosphatidate, platelet activating factor, arachidonic acid) involved in cell signaling (1–5). PC is also a component of pulmonary surfactant (6), serum lipoproteins (7), and bile (8). The importance of PC to cell physiology is highlighted by the fact that alterations in its metabolism are linked to programmed cell death (9–12) and oncogenic transformation (13–15).

In the yeast Saccharomyces cerevisiae, PC is synthesized by the CDP-choline branch of the Kennedy pathway and by the CDP-diacylglycerol pathway (reviewed in refs. 1,2,4,16). In the Kennedy pathway, PC is derived from choline via phosphocholine and CDP-choline whereas in the CDP-diacylglycerol pathway, PC is derived from CDP-diacylglycerol via the major phospholipids phosphatidylserine and phosphatidylethanolamine (1,2,4,16). When grown in

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\*This work was supported in part by United States Public Health Service, National Institutes of Health Grant GM-50679.

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1The abbreviations used is: PC, phosphatidylcholine
the presence of choline, wild type *S. cerevisiae* primarily synthesizes PC via the Kennedy pathway (17). However, when cells are grown in the absence of choline, PC is primarily synthesized via the CDP-diacylglycerol pathway (17). The PC synthesized via the CDP-diacylglycerol pathway is constantly metabolized to free choline and phosphatidate via phospholipase D (18,19). Consequently, the Kennedy pathway contributes to PC synthesis even when cells are not supplemented with choline (18,20–24). Analyses of mutants defective in PC synthesis indicate that the PC synthesized by both pathways is not functionally equivalent (4,25,26). The basis for these differences may be the varying molecular species of the PC synthesized by the two pathways (25,26).

The regulation of the Kennedy pathway for PC synthesis is important to overall lipid metabolism and cell physiology in *S. cerevisiae* and in higher eukaryotic organisms (3). The *CKI1*-encoded choline kinase (27,28) (ATP:choline phosphotransferase, EC 2.7.1.32) should play a pivotal role in its regulation since the enzyme catalyzes the committed step in the pathway (4,29,30). Choline kinase catalyzes the phosphorylation of choline with ATP to form phosphocholine and ADP (31). The deduced protein product of the *CKI1* gene contains conserved phosphotransferase and choline kinase motifs (32–34) that are involved in catalytic function (34–36) (Fig. 1). Understanding the regulation of choline kinase is emphasized by the fact that unregulated levels of this enzyme play a role in the generation of human tumors by *ras* oncogenes (37–40). In addition, choline kinase activity may be used as a marker for cancer (41,42) and the enzyme is a target for anticancer drug discovery (43–45).

Phosphorylation is a major mechanism by which enzymes are regulated (46,47), and indeed, the choline kinase from *S. cerevisiae* is regulated by phosphorylation (48). Choline kinase is phosphorylated on multiple serine residues in *vivo*, and some of this phosphorylation is mediated by protein kinase A (48). Protein kinase A phosphorylates and stimulates (~ 2-fold) choline kinase at Ser$^{30}$ and Ser$^{85}$, with the former site having the major effect on enzyme regulation (48,49). Moreover, phosphorylation at these sites stimulates PC synthesis via the Kennedy pathway. In the present work, we addressed the hypothesis that phosphorylation of choline kinase was also mediated by protein kinase C. Protein kinase C is a lipid-dependent protein kinase required for *S. cerevisiae* cell cycle (50–54) and plays a role maintaining cell wall integrity (55). The rationale for this hypothesis was based on the presence of potential protein kinase C target sites in the choline kinase enzyme (Fig. 1). We showed here that protein kinase C phosphorylated and stimulated choline kinase, and identified Ser$^{25}$ and Ser$^{30}$ as major sites of phosphorylation. We also showed a S25A mutation in choline kinase correlated with a decrease in PC synthesis by the Kennedy pathway.

**EXPERIMENTAL PROCEDURES**

**Materials—**

All chemicals were reagent grade. Difco Laboratories was the source of growth medium supplies. Nucleotides, ammonium reinecke, phenylmethylsulfonyl fluoride, benzamidine, aprotinin, leupeptin, pepstatin, choline, l-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin, standard phosphoamino acids, and bovine serum albumin were purchased from Sigma. Protein kinase C (rat brain) and protein kinase A (bovine heart) were purchased from Promega. Bio-Rad was the source of the protein assay reagent, electrophoresis reagents, and protein molecular mass markers. Protein A-Sepharose CL-4B beads, polyvinylidene difluoride membrane, the enhanced chemiluminescence Western blotting detection kit and [methyl-$^{14}$C] choline were purchased from GE Healthcare. Phospholipids were purchased from Avanti Polar Lipids. Silica Gel 60 thin-layer chromatography plates and cellulose thin layer glass plates were purchased from EM Science. Peptides were synthesized and purified commercially by Bio-Synthesis, Inc. Phosphocellulose filters were supplied by Pierce. [$\gamma$-$^{32}$P]ATP was purchased from PerkinElmer Life Sciences. Scintilllation counting supplies were from National
Diagnostics. Restriction enzymes, modifying enzymes, and Vent DNA polymerase were from New England Biolabs. Polymerase chain reaction and sequencing primers were prepared commercially by Genosys Biotechnologies, Inc. The QuikChange site-directed mutagenesis kit was purchased from Stratagene. The Yeastmaker yeast transformation system was from Clontech. The DNA size ladder used for agarose gel electrophoresis was from Life Technologies, Inc. The plasmid DNA purification and DNA gel extraction kits were from Qiagen, Inc.

**Strains and Growth Conditions**

The strains used in this work are listed in Table I. The growth and analysis of yeast were performed by standard methods (56,57). Yeast cultures were grown in synthetic complete medium (56) containing 2% glucose at 30 °C. Plasmid maintenance and amplifications were performed in *Escherichia coli* strain DH5α. Bacteria were cultured in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) at 37 °C. Ampicillin (100 μg/ml) was added to the growth medium to select bacterial cells that carried plasmids. Growth media were supplemented with either 2% (yeast) or 1.5% (*E. coli*) agar for growth on plates. Yeast growth in liquid media was monitored spectrophotometrically (A600 nm).

**DNA Manipulations, Amplification of DNA by PCR, and DNA Sequencing**

Standard methods were used to prepare genomic and plasmid DNA, to digest DNA with restriction enzymes, and to ligate DNA (57). Transformation of yeast (58,59) and *E. coli* (57) were performed by standard methods. PCR reactions were optimized as described by Innis and Gelfand (60). DNA sequencing reactions were performed by the dideoxy method using Taq polymerase (57) and analyzed with an automated DNA sequencer.

**Construction of Plasmids and Expression of Wild Type and Mutant CKI1 Alleles**

The plasmids used in this work are listed in Table I. The CKI1<sup>S25A</sup> (primers: 5'-GCAAGTTCCAGATCGAGTGCTCAAAGAAGACAT TC-3' and its complement) CKI1<sup>S25D</sup> (primers: 5'-GTCCAGATCGAGTGATCAAAGAAGAC-3' and its complement), and CKI1<sup>S37A</sup> (primers: 5'-CACGCCAACGTTCCGTCAAAGACTGATTAG-3' and its complement) alleles were constructed by PCR with the QuikChange site-directed mutagenesis kit using plasmid pYY264 (49) as the template. The CKI1<sup>S25A, S37A</sup> and CKI1<sup>S25A, S30A</sup> mutant alleles were constructed with the primers for the S37A and S30A mutations using plasmid pCK25A as the template, respectively. The correct mutations in the CKI1 alleles were confirmed by DNA sequencing. The *eki1Δ cki1Δ* mutant strain KS106 (61) was transformed with plasmids containing the wild type and mutant alleles of the *CKI1* gene.

**Preparation of Enzymes**

Cell extracts were prepared by disruption of cells with glass beads using a Mini-BeadBeater-8 (Biospec Products, Inc.) as described previously (49,62). The cell disruption buffer contained 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.3 M sucrose, 10 mM 2-mercaptoethanol, and a protease and phosphatase inhibitor cocktail. The protease and phosphatase inhibitor cocktail contained 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 10 mM NaF, and 5 mM β-glycerophosphate. The CKII-encoded choline kinase was expressed in Sf9 insect cells and purified to homogeneity from the cytosolic fraction by chromatography with Con A, Affi-Gel Blue, and Mono Q (28).

**Immunoprecipitation and Immunoblotting**

The IgG fraction of rabbit anti-choline kinase antibodies (49) was isolated from antisera by protein A-Sepharose chromatography (63) and used for immunoprecipitation and immunoblotting experiments. Cell extracts (0.5 mg protein) were incubated for 1 h with 10
μg of anti-choline kinase antibodies in a total volume of 0.5 ml followed by incubation with 100 μl of protein A-Sepharose CL-4B beads (10% slurry, w/v) for 1 h at 4 °C. Immune complexes were collected by centrifugation at 1,500 x g for 30 s and washed three times with phosphorylation reaction buffer (50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 0.375 mM EDTA, 0.375 mM EGTA, and 1.7 mM CaCl₂). Following the washing steps, the buffer was removed by aspiration and the choline kinase attached to the protein A-Sepharose CL-4B beads was used as substrate for protein kinase C phosphorylation. The reaction mixtures were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membrane was probed with anti-choline kinase antibodies (0.6 μg/ml) as described previously (63). The choline kinase protein was detected on immunoblots using the ECF Western Blotting chemifluorescent detection kit as described by the manufacturer. Fluorescent signals on the immunoblots were acquired by FluorImaging analysis. The relative densities of the proteins were analyzed using ImageQuant software. Immunoblot signals were in the linear range of detectability.

**Phosphorylation Reactions**

Pure choline kinase, immunoprecipitated choline kinase and choline kinase synthetic peptides were phosphorylated with rat brain protein kinase C. This enzyme preparation contains a mixture of the α, β, and γ isoforms of the enzyme. We used the rat brain protein kinase C in our studies because the *S. cerevisiae* protein kinase C (64,65) has catalytic properties characteristic of the α, β, and γ isoforms of the rat brain enzyme (50,66). In addition, the rat brain enzyme has been shown to phosphorylate other yeast proteins with the same efficiency as a partially purified preparation of yeast protein kinase C (67–69).

Phosphorylation reactions were performed in a total volume of 25 μl at 30 °C. Choline kinase was incubated for 10 min with 50 mM Tris-HCl (pH 8.0), 50 μM [γ-³²P]ATP (4 μCi/nmol), 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 0.375 mM EDTA, 0.375 mM EGTA, 1.7 mM CaCl₂, 20 μM diacylglycerol, 50 μM phosphatidylserine, and the indicated amounts of protein kinase C. At the end of the phosphorylation reactions, samples were treated with 4x Laemmli’s sample buffer (70), followed by SDS-PAGE and transfer to polyvinylidene difluoride membrane. The phosphorylated enzyme was visualized by phosphorimaging and the extent of phosphorylation was quantified using ImageQuant software. To measure the effect of phosphorylation on choline kinase activity, the protein kinase C phosphorylation reactions were performed with unlabeled ATP. Following incubation with protein kinase C, the reaction mixtures were diluted 2-fold and choline kinase activity was measured. Phosphorylation reactions containing synthetic peptides were terminated by spotting the reaction mixture onto phosphocellulose filters. The filters were washed with 75 mM phosphoric acid and then subjected to scintillation counting. Phosphorylation of choline kinase with protein kinase A was performed as described previously (48,49).

**Phosphoamino Acid Analysis and Two-dimensional Peptide Mapping**

³²P-labeled choline kinase was extracted from SDS polyacrylamide gel slices and subjected to acid hydrolysis with 6 N HCl as described by Yang and Carman (71). Hydrolysates were dried in vacuo and applied to 0.1 mm cellulose thin-layer chromatography plates with 2.5 μg phosphoserine, 2.5 μg phosphothreonine and 5 μg phosphotyrosine as carrier phosphoamino acids in water. Phosphoamino acids were separated by two-dimensional electrophoresis (72). Following electrophoresis, the plates were dried, sprayed with 0.25% ninhydrin in acetone to visualize carrier phosphoamino acids, and subjected to phosphorimaging analysis.

Polyvinylidene difluoride membrane slices containing ³²P-labeled choline kinase proteins were subjected to digestion with L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin and two-dimensional peptide mapping analysis (73). Electrophoresis (1% ammonium...
bicarbonate buffer at 1000 volts for 45 min) and ascending chromatography (n-butyl alcohol/glacial acetic acid/pyridine/water, 10:3:12:15 for 8 h) were performed on cellulose thin-layer glass plates. Dried plates were then subjected to phosphorimaging analysis.

**Choline Kinase Assay and Protein Determination—**

Choline kinase activity was measured in a reaction mixture that contained 67 mM glycine-NaOH buffer (pH 9.5), 5 mM [methyl-14C]choline (2,000 cpm/nmol), 5 mM ATP, 10 mM MgSO4, 1.3 mM dithiothreitol, and enzyme protein in a final volume of 30 μl (28). The radiolabeled product phosphocholine was separated from the radiolabeled substrate by precipitation of choline as a reineckate salt (74). Alternatively, the phosphocholine was separated from choline by thin-layer chromatography on silica gel plates using the solvent system methanol/0.5% sodium chloride/ammonium hydroxide (50:50:1, v/v) (75). The average standard deviation of the enzyme assays (performed in triplicate) was +5%. Enzyme reactions were linear with time and protein concentration. A unit of choline kinase activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product/min. Specific activity was defined as units per mg of protein. Protein concentration was determined by the Coomassie blue dye-binding assay of Bradford (76) using bovine serum albumin as the standard.

**Labeling and Analysis of PC—**

Exponential phase cki1Δ eki1Δ cells containing the wild type and mutant choline kinase enzymes were labeled for 30 min with 100 μM [methyl-14C]choline (0.2 μCi/ml). Following the labeling period, phospholipids were extracted from the cells (77,78) and PC was analyzed on silica gel thin-layer chromatography plates using the solvent system chloroform/methanol/ammonium hydroxide/water (70:30:2:2, v/v). Radiolabeled PC was visualized by phosphorimaging analysis. The position of the labeled PC on chromatography plates was compared with standard PC after exposure to iodine vapor. The amount of labeled PC was determined by liquid scintillation counting.

**Analyses of Data—**

Kinetic data were analyzed with the EZ-FIT enzyme kinetic model-fitting program (79). Statistical analyses were performed with SigmaPlot software.

**RESULTS**

**Phosphorylation of Pure Choline Kinase by Protein Kinase C—**

To determine if choline kinase was a target for protein kinase C phosphorylation, we examined whether protein kinase C catalyzed the incorporation of the γ phosphate of 32P-labeled ATP into purified choline kinase. After the reaction, samples were subjected to SDS-PAGE followed by transfer to polyvinylidene difluoride membrane. Phosphorimaging analysis of the polyvinylidene difluoride membrane showed that choline kinase was a substrate for protein kinase C (Fig. 2). The phosphorylation of choline kinase was dependent on the concentration of protein kinase C (Fig. 2A). Analysis of the kinetic properties of protein kinase C activity showed that the enzyme followed positive cooperative kinetics (80) with respect to the concentrations of choline kinase (Fig. 2B) and ATP (Fig. 2C). The data were analyzed according to the Hill equation using the EZ-FIT Enzyme Kinetic Model Fitting Program (79). The $K_m$ values for choline kinase and ATP were 27 μg/ml and 15 μM, respectively, and the Hill numbers for these substrates were 2.3 and 1.8, respectively. The cooperative kinetic behavior of protein kinase C activity towards choline kinase may be a reflection of the oligomeric forms of the choline kinase enzyme (28).
To examine which amino acid residue(s) of choline kinase was a target for phosphorylation, choline kinase was phosphorylated with protein kinase C, and the 32P-labeled enzyme was subjected to phosphoamino acid analysis. Protein kinase C phosphorylated choline kinase on a serine residue (Fig. 3A). Phosphopeptide mapping analysis of the phosphorylated choline kinase showed several phosphopeptides indicating that the enzyme was phosphorylated on multiple serine residues (Fig. 3B).

The effect of protein kinase C phosphorylation on choline kinase activity was examined. For this experiment, the pure choline kinase enzyme was phosphorylated with protein kinase C and unlabeled ATP. Following the phosphorylation reaction, choline kinase activity was measured by following the formation of radioactive phosphocholine from [methyl-14C]choline. Phosphorylation by protein kinase C resulted in a dose-dependent stimulation (1.6-fold) of choline kinase activity (Fig. 4). This extent of stimulation might be an underestimate of the overall effect protein kinase C has on activity because the purified choline kinase is already partially phosphorylated (48).

### Choline Kinase Synthetic Peptides Containing a Protein Kinase C Sequence Motif Are Substrates for Protein Kinase C—

The deduced sequence of choline kinase has five potential serine (Ser<sup>9</sup>, Ser<sup>25</sup>, Ser<sup>37</sup>, Ser<sup>198</sup>, and Ser<sup>236</sup>) phosphorylation sites within a protein kinase C sequence motif (Fig. 1). Peptides (S9, S25, S37, S198, and S236) containing these serine target sites were synthesized based on the amino acid sequence of choline kinase (Table II). We examined whether these peptides could serve as substrates for protein kinase C. All five peptides served as substrates, with the greatest activity using the S25 peptide followed by the S37 peptide (Table II). Peptides (A9, A25, A37, A198, and A236) were also synthesized where the serine residues within the protein kinase C sequence motif were changed to alanine residues. These peptides did not serve as substrates for protein kinase C. Thus, the serine residue contained within the protein kinase C motif of the S9, S25, S37, S198, and S236 peptides was the target site for phosphorylation.

A kinetic analysis was performed to further characterize the choline kinase synthetic peptides as substrates for protein kinase C activity. The dependence of protein kinase C activity on each of the peptide substrates followed saturation kinetics. The data were analyzed according to the Michaelis-Menten equation and the kinetic constants are summarized in Table II. Based on the values of $V_{\text{max}}$, $K_m$, and $V_{\text{max}}/K_m$, the S25 peptide was by far the best substrate followed by the S37 peptide. These data indicated that Ser<sup>25</sup> and Ser<sup>37</sup> might be major protein kinase C phosphorylation sites in the choline kinase enzyme.

### Effects of Protein Kinase C Site Mutations on the Phosphorylation of Choline Kinase by Protein Kinase C and by Protein Kinase A—

Mutagenesis of Ser<sup>25</sup> and Ser<sup>37</sup> within the choline kinase was performed to further test the hypothesis that these sites are targets for protein kinase C. CKII alleles with serine to alanine (S25A and S37A) mutations were constructed by site-directed mutagenesis. These protein kinase C phosphorylation site mutant alleles were expressed from a multicopy plasmid in a cki1Δ eki1Δ mutant to obviate any effects due to the choline kinase activities expressed by the native CKII (27) and EKII (61) genes. A multicopy plasmid was used for increased expression to facilitate isolation of the mutant enzymes by immunoprecipitation from cell extracts. Immunoblot analysis showed that the mutations did not affect the expression of the choline kinase enzyme.

In control experiments, we showed that the phosphorylation of the immunoprecipitated wild type choline kinase was dependent on both the concentration of protein kinase C and the time of the reaction. The immunoprecipitated mutant choline kinase enzymes were incubated with...
protein kinase C and 32P-labeled ATP. After the phosphorylation reactions, samples were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membrane, and then analyzed for radioactive label incorporated into choline kinase. The extent of phosphorylation of the S25A and S37A mutant enzymes was reduced by 60% and 20%, respectively, when compared with the wild type enzyme. We also constructed a S25A, S37A mutant choline kinase enzyme and tested it as a substrate for protein kinase C. The effect of the S25A, S37A mutation on the protein kinase C phosphorylation of the enzyme was similar to that of the S25A mutation.

The immunoprecipitated wild type and protein kinase C phosphorylation site mutant choline kinase enzymes were also subject to phosphorylation with protein kinase A (48,49). Following a 10 min incubation period, the extent of phosphorylation was examined as described above. The S25A and S37A mutations did not affect the phosphorylation of the choline kinase by protein kinase A.

**Effects of Protein Kinase A Site Mutations on the Phosphorylation of Choline Kinase by Protein Kinase C—**

Ser\(^{30}\) and Ser\(^{85}\) are target sites for protein kinase A phosphorylation in the choline kinase enzyme (49). We questioned whether the S30A and S85A mutations affected phosphorylation of the enzyme by protein kinase C. The S30A mutation caused a decrease in protein kinase C phosphorylation of choline kinase by 46% whereas the S85A mutation did not affect the phosphorylation of the enzyme.

**A Choline Kinase Synthetic Peptide Containing Amino Acid Residue Ser\(^{30}\) is a Substrate for Protein Kinase C—**

The choline kinase peptides S30 and S85 (Table II), which contain amino acid residues Ser\(^{30}\) and Ser\(^{85}\), respectively, are substrates for protein kinase A phosphorylation (49). We examined whether these peptides could also serve as substrates for protein kinase C. The S30 peptide was a relatively good substrate for protein kinase C phosphorylation whereas the S85 peptide did not serve as a substrate for this kinase (Table II). The A30 peptide, which has an alanine residue substituted for the serine within the protein kinase A sequence motif, was a poor substrate for protein kinase C. This result indicated that the serine residue within the protein kinase A sequence motif of peptide S30 was the target site for protein kinase C phosphorylation.

The phosphorylation of the S30 peptide by protein kinase C followed saturation kinetics, and the kinetic constants are presented in Table II. Based on the \(V_{\text{max}}\) value, the S30 peptide was almost as good of a substrate for protein kinase C as the S25 peptide (Table II). However, the \(K_m\) value for the S30 peptide was 4.7-fold higher than the \(K_m\) value for the S25 peptide. Consequently, the specificity constant (\(V_{\text{max}}/K_m\)) for the S30 peptide was 5.8-fold lower than that of the S25 peptide (Table II). The \(K_m\) value for the S30 peptide for protein kinase A phosphorylation is 19.5 \(\mu\)M (49). Thus, based on this assay, Ser\(^{30}\) was a better target site for protein kinase A phosphorylation when compared with protein kinase C.

**Effects of S25A, S30A, and S25A, S30A Mutations on the Phosphopeptide Map of Choline Kinase Phosphorylated by Protein Kinase C—**

The effects of the S25A and S30A mutations on the phosphorylation of choline kinase by protein kinase C were examined in more detail. The phosphorylation of the S25A and S30A mutant choline kinase enzymes was time-dependent, but at the 20 min time interval the extent of phosphorylation was reduced 60% and 40%, respectively, when compared with the wild type enzyme (Fig. 5). A S25A, S30A mutant was also constructed, expressed, and examined for its phosphorylation by protein kinase C. The phosphorylation of this double mutant was further reduced (70%) when compared with the single phosphorylation site mutants (Fig. 5).
We examined the effects of the S25A and S30A mutations on the phosphopeptide map of the choline kinase. Choline kinase was immunoprecipitated from cells expressing the wild type, S25A, or S30A mutant enzymes. The immunoprecipitated proteins were phosphorylated with protein kinase C and $^{32}$P-labeled ATP, and subjected to two-dimensional phosphopeptide mapping analysis. The phosphopeptide map of the immunoprecipitated choline kinase phosphorylated with protein kinase C was very similar to that of the pure choline kinase phosphorylated with protein kinase C (compare Fig. 6A with Fig. 3B). The phosphopeptides labeled 3 and 4 in the phosphopeptide map of the wild type choline kinase enzyme (Fig. 6A) were greatly reduced in the map of the S25A mutant enzyme (Fig. 6B). The S30A mutation resulted in the loss of phosphopeptide 1 (Fig. 6C). In the phosphopeptide map of the S25A, S30A mutant, phosphopeptide 1 was absent and phosphopeptides 3 and 4 were greatly reduced (Fig. 6D). These data indicated that Ser$_{25}$ was contained in phosphopeptides 3 and 4, and that Ser$_{30}$ was contained in phosphopeptide 1. An explanation for the presence of Ser$_{25}$ in two phosphopeptides might be partial cleavage of the phosphorylated enzyme by trypsin digestion.

**Effects of the S25A and S25D Mutations on Choline Kinase Protein and Activity, and on the Synthesis of PC by the Kennedy Pathway—**

Immunoblot analysis of the cell extract derived from cki1Δ eki1Δ cells expressing the wild type choline kinase showed that the enzyme migrated as a doublet upon SDS-PAGE (Fig. 7A). This choline kinase doublet is also observed when the enzyme is expressed in Sf9 insect cells (28). However, with the S25A mutant enzyme, the intensity of the upper (slower migrating) band was reduced when compared with the lower (faster migrating) band (Fig. 7A). An electrophoretic mobility shift is a characteristic property exhibited by some phosphorylated enzymes (81,82). We constructed and expressed a choline kinase with a Ser$_{25}$ to Asp (S25D) mutation to examine the effect of a phosphorylation mimic on the enzyme. Immunoblot analysis showed that the S25D mutant enzyme migrated as a doublet like the wild type enzyme (Fig. 7A). These data indicated that a population of phosphorylated and dephosphorylated forms of choline kinase existed in vivo.

The effects of the S25A and S25D mutations on the specific activity of choline kinase were examined. Cell extracts were prepared from cki1Δ eki1Δ cells expressing the mutant enzymes and assayed for choline kinase activity. The specific activity of the S25A mutant choline kinase was reduced by 32% when compared with the wild type enzyme (Fig. 7B). The specific activity of the S25D mutant enzyme was 14% higher than that of the wild type enzyme (Fig. 7B).

To examine the effect of phosphorylation of choline kinase at Ser$_{25}$ on the synthesis of PC, cki1Δ eki1Δ cells expressing the S25A mutant enzyme were pulse-labeled (30 min) with 100 μM [methyl-$^{14}$C]choline. Labeled choline is only incorporated into PC that is synthesized by the Kennedy pathway (17,23,24). Following the labeling period, phospholipids were extracted from cells, and PC was analyzed by one-dimensional thin-layer chromatography. The incorporation of the labeled choline into PC in cells expressing the S25A mutant enzyme was reduced by 55% when compared with cells expressing the wild type enzyme (Fig. 7C). We also examined the effect of the S25D mutation, which mimics the phosphorylation at Ser$_{25}$, on PC synthesis. The S25D mutation in choline kinase resulted in a 44% increase in the incorporation of labeled choline into PC (Fig. 7C). Overall, the synthesis of PC in cells expressing the S25D mutant choline kinase was 3.2-fold greater than that of cells expressing the S25A mutant enzyme.

**DISCUSSION**

The CKII-encoded choline kinase is an important regulatory enzyme that catalyzes the committed step in PC synthesis via the CDP-choline branch of the Kennedy pathway in S. cerevisiae (3,4,17,29,30). Covalent modification by phosphorylation is an important
mechanism by which the activity of an enzyme (and a metabolic pathway) may be regulated (46,47). Previous studies have shown that the yeast choline kinase is phosphorylated and regulated by protein kinase A (48,49). Phosphopeptide mapping analysis of choline kinase phosphorylated in vivo and phosphorylated in vitro by protein kinase A indicate that additional protein kinases are responsible for the phosphorylation of choline kinase (48). In the present work, we addressed the hypothesis that choline kinase phosphorylation was also mediated by protein kinase C. In vitro, protein kinase C phosphorylated choline kinase on serine residues resulting in the stimulation of activity. The phosphorylation of choline kinase was time- and dose-dependent, and dependent on the concentrations of choline kinase and ATP. These results indicated that the yeast choline kinase was a substrate for protein kinase C. To our knowledge, this is the first report of protein kinase C phosphorylation of a choline kinase from any organism.

Identification of the protein kinase C phosphorylation sites in choline kinase was addressed to gain information about the physiological relevance of this phosphorylation. Through a computer analysis, we identified five potential protein kinase C target sites in choline kinase (Fig. 1). Synthetic peptides with sequences for these sites were synthesized and examined for their ability to serve as substrates for protein kinase C. While all five peptides served as substrates, the peptides containing a protein kinase C phosphorylation motif at Ser^{25} and Ser^{37} were the best. This assay provided confidence that these residues might be major phosphorylation sites for protein kinase C. To provide support for this hypothesis, S25A and S37A mutations in choline kinase were constructed and expressed in ckIΔ ekiΔ cells. The S25A mutation had a major effect on the ability of choline kinase to be phosphorylated by protein kinase C. The extent of phosphorylation of this mutant was reduced by 60% when compared with the wild type enzyme. Moreover, phosphopeptide-mapping analysis of protein kinase C-phosphorylated choline kinase showed that two distinct phosphopeptides present in the wild type enzyme were greatly reduced in the S25A mutant enzyme. These data provided strong evidence that Ser^{25} was a specific target for protein kinase C phosphorylation. Extracts derived from cells expressing the S25A mutant exhibited reduced choline kinase activity. Thus, phosphorylation at Ser^{25} contributes to the activation of choline kinase activity. These results correlated with the stimulation of choline kinase activity upon protein kinase C phosphorylation in vitro.

The S37A mutant did not have a major effect on the protein kinase C phosphorylation of choline kinase. The phosphopeptide map of the S37A mutant was indistinguishable from that of the wild type enzyme, and the phosphopeptide map of the S25A, S37A mutant enzyme did not differ from that of the S25A mutant (data not shown). Accordingly, we did not consider Ser^{37} as a major protein kinase C phosphorylation site in choline kinase.

We addressed the question of whether the phosphorylation of choline kinase by protein kinase C affected the phosphorylation of the enzyme by protein kinase A, and vice versa (i.e., hierarchal phosphorylation (83,84)). Whereas the S25A (protein kinase C site) mutation did not affect the phosphorylation of choline kinase by protein kinase A, the S30A (protein kinase A site) mutation caused a 46% reduction in enzyme phosphorylation by protein kinase C. This effect could be attributed to a decrease in the rate of phosphorylation. An explanation for the reduced protein kinase C phosphorylation of the S30A mutant choline kinase was that Ser^{30} was a target site for protein kinases A and C. The choline kinase synthetic peptide containing Ser^{30}, which is a substrate for protein kinase A (49), was also a substrate for protein kinase C. Moreover, a major phosphopeptide in the phosphopeptide map of the wild type protein kinase C-phosphorylated enzyme was absent in the phosphopeptide map of the S30A mutant enzyme. This same phosphopeptide results from the protein kinase A phosphorylation of choline kinase (49). That one site is phosphorylated by multiple protein kinases is not uncommon (84–86).
For example, the yeast URA7-encoded CTP synthetase is phosphorylated at Ser\(^{424}\) by both protein kinase A and protein kinase C (86,87).

The choline kinase reaction in *S. cerevisiae* is an important regulatory step in the synthesis of PC via the Kennedy pathway (17). Accordingly, we questioned the physiological relevance of the protein kinase C phosphorylation of Ser\(^{25}\) with respect to PC synthesis in cells bearing the S25A and S25D mutant choline kinase enzymes. The *in vivo* labeling studies showed that cells bearing the S25A mutation exhibited a decrease in PC synthesis whereas cells bearing the S25D mutation exhibited an increase in PC synthesis. These data supported the conclusion that activation of choline kinase activity by protein kinase C phosphorylation at Ser\(^{25}\) resulted in the stimulation of PC synthesis via the Kennedy pathway. However, the regulation of choline kinase activity by phosphorylation is complex. The enzyme is also phosphorylated by protein kinase A, and it may be phosphorylated by additional protein kinases. Thus, we cannot rule out other phosphorylation or regulatory events that might affect PC synthesis in cells carrying the S25A and S25D mutant choline kinase enzymes.

Previous studies have shown that the S30A (protein kinase A site) mutation in choline kinase results in a decrease in PC synthesis (49). This observation has lead to the conclusion that protein kinase A phosphorylation at Ser\(^{30}\) stimulates PC synthesis via the Kennedy pathway (49). As shown in the present work, the phosphorylation of choline kinase at Ser\(^{30}\) was also mediated by protein kinase C. Thus, the phosphorylation at Ser\(^{30}\) by both protein kinases should play a role in the regulation of PC synthesis via the Kennedy pathway. Additional studies will be required to determine the physiological conditions that protein kinase C phosphorylates choline kinase at Ser\(^{25}\) and Ser\(^{30}\), and the conditions that protein kinase A phosphorylates the enzyme at Ser\(^{30}\).

In mammalian cells, protein kinase C plays a central role in the transduction of lipid second messengers generated by receptor-mediated hydrolysis of membrane phospholipids (e.g., PC and phosphatidylinositol 4,5-bisphosphate) (88–90). Phosphorylation of choline kinase by protein kinase C in *S. cerevisiae* may represent a mechanism by which lipid signaling transduction pathways are coordinately regulated to PC synthesis and cell growth.

**Acknowledgements**

We thank Gil-Soo Han for helpful suggestions during the course of this work.

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J Biol Chem. Author manuscript; available in PMC 2006 February 28.
FIG. 1. Domain structure of choline kinase.
The diagram shows the positions of the phosphotransferase (P-transferase) and choline kinase motifs, and protein kinase A (PKA) target sites in the choline kinase protein sequence. The putative (indicated by question marks) protein kinase C (PKC) target sites are also indicated. The numbers on the top of the diagram indicate amino acid positions.
FIG. 2. Phosphorylation of pure choline kinase by protein kinase C.

Panel A, pure choline kinase (50 μg/ml) was incubated with the indicated amounts of protein kinase C and [γ-32P]ATP for 10 min. Panel B, protein kinase C (0.1 U/ml) and [γ-32P]ATP were incubated with the indicated concentrations of pure choline kinase for 10 min. Panel C, protein kinase C (0.1 U/ml) and pure choline kinase (50 μg/ml) were incubated with the indicated concentrations of [γ-32P]ATP for 10 min. Following the phosphorylation incubations, samples were subjected to SDS-PAGE and then transferred to polyvinylidene difluoride membrane. The phosphorylated proteins were subjected to phosphorimaging analysis and the relative amounts of phosphate incorporated were quantified using ImageQuant software. Portions of the images with the phosphorylated choline kinase are shown above each panel. The data are representative of three independent experiments.
FIG. 3. Phosphoamino acid and phosphopeptide mapping analyses of pure choline kinase phosphorylated by protein kinase C.

Pure choline kinase was phosphorylated with protein kinase C (0.1 U/ml) and [γ-32P]ATP for 10 min. Panel A, samples were subjected to SDS-PAGE and gel slices containing 32P-labeled choline kinase were subjected to phosphoamino acid analysis. The positions of the carrier standard phosphoamino acids phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) are indicated in the figure. Panel B, samples were subjected to SDS-PAGE followed by transfer to polyvinylidene difluoride membrane. Membrane pieces containing 32P-labeled choline kinase were digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin. The resulting peptides were separated on cellulose thin layer plates by electrophoresis (from left to right) in the first dimension and by chromatography (from bottom to top) in the second dimension. The data shown in panels A and B were representative of three independent experiments.
FIG. 4. Effect of protein kinase C phosphorylation on choline kinase activity.
Pure choline kinase was phosphorylated with the indicated concentrations of protein kinase C with unlabeled ATP for 15 min. Following the phosphorylation, samples were diluted 2-fold, and choline kinase activity was measured with 0.4 mM [methyl-14C]choline and 0.15 mM ATP.
FIG. 5. Effects of the S25A, S30A, and S25A, S30A mutations on the time-dependent phosphorylation of choline kinase by protein kinase C.

Choline kinase was immunoprecipitated from cell extracts (500 μg) of cki1Δ eki1Δ cells expressing wild type (WT) and the indicated phosphorylation site mutant choline kinase enzymes using 10 μg anti-choline kinase antibodies. The immunoprecipitates were incubated with protein kinase C and [γ-32P]ATP for the indicated time intervals. Following the phosphorylation reactions, the immunoprecipitates were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane. The phosphorylated proteins were subjected to phosphorimaging analysis and the relative amounts of phosphate incorporated were quantified using ImageQuant software. The data were normalized to the amount of choline kinase enzymes in the immunoprecipitates as determined by immunoblot analysis. The data are representative of two independent experiments.
FIG. 6. Effects of the S25A, S30A, and S25A, S30A mutations on the phosphopeptide map of choline kinase phosphorylated by protein kinase C.

Choline kinase was immunoprecipitated from cell extracts (500 μg) of cki1Δ eki1Δ cells expressing wild type (WT) (panel A), S25A (panel B), S30A (panel C), and S25A, S30A (panel D) mutant choline kinase enzymes using 10 μg anti-choline kinase antibodies. The immunoprecipitates were incubated with protein kinase C and [γ-32P]ATP for 10 min, subjected to SDS-PAGE, and transferred to polyvinylidene difluoride membrane. The 32P-labeled proteins on the polyvinylidene difluoride membrane were subjected to phosphopeptide mapping analysis as described in the legend to Fig. 3. The positions of the phosphopeptides that were absent (or had a reduced signal) in the S25A, S30A, and S25A, S30A mutant choline kinase enzymes but were present in the wild type enzyme phosphorylated by protein kinase C are indicated by the ellipses in the figure. The data are representative of three independent experiments.
Fig. 7. Choline kinase protein and activity levels in cells bearing the S25A and S25D mutations, and the effects of these mutations on the synthesis of PC via the CDP-choline pathway.

ckilΔ ekiΔ cells expressing the wild type (WT) and the indicated S25A and S25D mutant choline kinase enzymes were grown to the exponential phase of growth. Cell extracts (50 μg) were prepared and assayed for choline kinase protein by immunoblot analysis (panel A) and for activity (panel B). A portion of the immunoblot showing the choline kinase doublet is presented in the figure. Panel C, the exponential phase cells were incubated with 100 μM [methyl-14C]choline (0.2 μCi/ml) for 30 min to label PC via the CDP-choline pathway. The chloroform-soluble fraction of the cells was extracted and analyzed for PC by one-dimensional thin-layer chromatography.
### TABLE I

Strains and plasmids used in this work

| Strain or plasmid | Genotype or relevant characteristics | Source or Ref. |
|-------------------|-------------------------------------|---------------|
| **E. coli**       |                                     |               |
| DH5α              | F<sup>−</sup> <small>φ<sub>80</sub></small> <small>λ<sub>DE3</sub></small> <small>lacZ<sub>ΔM15</sub></small> <small>ΔlacZYA-argF</small>:<small>U169</small> <small>deoR</small> <small>recA1</small> <small>endA1</small> <small>hsdR17</small> <small>(r<sub>K</sub> m<sub>K</sub>)</small> <small>phoA</small> <small>supE44</small> <small>λ<sub>thi-1</sub></small> <small>gyrA96</small> <small>relA1</small> | (57) |
| **S. cerevisiae** |                                     |               |
| KS106             | MATα ade2-1 can1-100 his3-11,15 leu2-3 112, trp1-1 ura3-1 ckl1Δ::HIS3 ckl1Δ::TRP1 | (61) |
| **Plasmids**      |                                     |               |
| Yep351            | Multicopy E. coli/yeast shuttle vector containing LEU2 | (91) |
| pYY264            | CKII derivative of Yep351           | (49) |
| pYY265            | CKI<sup>385A</sup> derivative of pYY264 | (49) |
| pYY266            | CKI<sup>385A</sup> derivative of pYY264 | (49) |
| pCK25A            | CKI<sup>385A</sup> derivative of pYY264 | This study |
| pCK25D            | CKI<sup>387A</sup> derivative of pYY264 | This study |
| pCK37A            | CKI<sup>387A</sup> derivative of pYY264 | This study |
| pCK25A/37A        | CKI<sup>385A,387A</sup> derivative of pCK25A | This study |
| pCK25A/30A        | CKI<sup>385A,390A</sup> derivative of pCK25A | This study |

*J Biol Chem. Author manuscript; available in PMC 2006 February 28.*
**TABLE II**
Kinetic constants for protein kinase C using choline kinase synthetic peptides

| Peptide       | Sequence | $V_{\text{max(app)}}$ mol/min/mg p | $K_{\text{m(app)}}$ mM | $V_{\text{max(app)}}/K_{\text{m(app)}}$ μmol/min/mg |
|---------------|----------|-----------------------------------|------------------------|-----------------------------------------------|
| Protein kinase C site |          |                                   |                        |                                              |
| S9           | SRPGS$^{\text{9}}$VRSYS | 0.5 ± 0.03                        | 1.2 ± 0.20             | 0.4                                          |
| S25          | SRSS$^{\text{25}}$QRRHS | 7.0 ± 0.30                        | 0.4 ± 0.04             | 17.5                                         |
| S37          | RQR$^{\text{37}}$LSQRLIR | 2.8 ± 0.20                        | 0.8 ± 0.10             | 3.5                                          |
| S198         | LARLS$^{\text{198}}$LKNIQ | 0.7 ± 0.04                        | 1.6 ± 0.20             | 0.4                                          |
| S236         | NWKNS$^{\text{236}}$QRIAR | 1.6 ± 0.90                        | 7.2 ± 5.00             | 0.2                                          |
| Protein kinase A site |        |                                   |                        |                                              |
| S30          | SQRRHS$^{\text{30}}$LTOQ | 5.7 ± 0.40                        | 1.9 ± 0.30             | 3.0                                          |
| S85          | GPRRAS$^{\text{85}}$ATDV | NA$^{\text{a}}$                   |                        |                                              |

$^{\text{a}}$NA, no activity