Phosphorylation of *Saccharomyces cerevisiae* Choline Kinase on Ser^{30} and Ser^{85} by Protein Kinase A Regulates Phosphatidylcholine Synthesis by the CDP-choline Pathway*

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The *Saccharomyces cerevisiae* CKI-encoded choline kinase is phosphorylated on a serine residue and stimulated by protein kinase A. We examined the hypothesis that amino acids Ser^{30} and Ser^{85} contained in a protein kinase A sequence motif in choline kinase are target sites for protein kinase A. The synthetic peptides SQR-RHSLTRQ (V_{max}/K_{m} = 10.8 μmol min^{-1} mol^{-1} mg^{-1}) and GPRRASATDV (V_{max}/K_{m} = 0.15 μmol min^{-1} mol^{-1} mg^{-1}) containing the protein kinase A motif for Ser^{30} and Ser^{85}, respectively, within the choline kinase protein were substrates for protein kinase A. Choline kinase with Ser^{30} to Ala (S30A) and Ser^{85} to Ala (S85A) mutations were constructed alone and in combination by site-directed mutagenesis and expressed in a *chklΔ eklΔ* double mutant that lacks choline kinase activity. The mutant enzymes were expressed normally, but the specific activity of choline kinase in cells expressing the S30A, S85A, and S30A,S85A mutant enzymes was reduced by 44, 8, and 60%, respectively, when compared with the control. In *in vivo* labeling experiments showed that the extent of phosphorylation of the S30A, S85A, and S30A,S85A mutant enzymes was reduced by 70, 17, and 83%, respectively. Phosphorylation of the S30A, S85A, and S30A,S85A mutant enzymes by protein kinase A in *vitro* was reduced by 60, 7, and 96%, respectively, and peptide mapping analysis of the mutant enzymes confirmed the phosphorylation sites in the enzyme. The incorporation of ^3H^-labeled choline into phosphocholine and phosphatidylcholine in cells bearing the S30A, S85A, and S30A,S85A mutant enzymes was reduced by 56, 27, and 81%, respectively, and by 58, 33, and 84%, respectively, when compared with control cells. These data supported the conclusion that phosphorylation of choline kinase on Ser^{30} and Ser^{85} by protein kinase A regulates PC synthesis by the CDP-choline pathway.

PC^1 is the most abundant phospholipid in eukaryotic organisms (1–4). It serves as a major structural component of cellular membranes (1–4), pulmonary surfactant (5), and platelet-activating factor, arachidonic acid (8). PC is synthesized by two major pathways: the CDP-choline (Kennedy) pathway and the three-step methylation of PE (1–4) (Fig. 1). In mammalian cells, PC is primarily synthesized via the CDP-choline pathway, whereas in the yeast *Saccharomyces cerevisiae*, PE methylation is the primary route of synthesis (8). In *S. cerevisiae*, PE is derived from PS, which is synthesized from CDP-DAG and serine (i.e. CDP-DAG pathway) (1) (3, 4). Both pathways play important roles in the growth and metabolism of higher and lower eukaryotic organisms (8).

Choline kinase (ATP:choline phosphotransferase; EC 2.7.1.32) is a cytosolic enzyme that catalyzes the committed step in the synthesis of PC by the CDP-choline pathway (9). The enzyme catalyzes the phosphorylation of choline with ATP to form phosphocholine and ADP (Fig. 1) (9). Genes encoding mammalian and yeast forms of choline kinase have been isolated (10–13), and various forms of the enzyme have been purified (14–17). The need to understand 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 (18–21). Moreover, methods are being developed where choline kinase activity is used as a marker for cancer (22, 23) and the enzyme is a target for anticancer drug discovery (24–26).

Because of its tractable genetics and ease of molecular manipulation, *S. cerevisiae* serves as an excellent eukaryotic model to study the regulation of choline kinase. The enzyme is encoded by the *CKI1* gene (13). Its deduced protein product contains a conserved phosphotransferase consensus sequence (27) (Fig. 2) believed to be involved in catalytic function (28, 29). The *CKI1* gene is not essential in *S. cerevisiae* (13) because PC is also synthesized by PE methylation (3, 4, 30). Nevertheless, choline kinase and the CDP-choline pathway become essential for PC synthesis when enzymes in the CDP-DAG pathway are defective (3, 4, 30). Indeed, mutants defective in the synthesis of PS, PE, or PC are choline auxotrophs (3, 4, 30). The expression of choline kinase is regulated by growth phase and by supplementation with water-soluble phospholipid precursors (28). Choline kinase mRNA and protein levels are highest in exponential phase and decline in the stationary phase (28). Similar to other phospholipid synthetic enzymes (4), choline kinase is repressed by the addition of inositol and choline to the growth medium (28).

Yeast choline kinase is also regulated by biochemical mechanisms. Studies with purified enzyme have shown that its substrate ATP and its product ADP allosterically regulate ac-
S85A mutations in choline kinase exhibited defects in PC syn-
nase A phosphorylation and show that cells bearing S30A and
identification of Ser30 and Ser85 as target sites of protein ki-
the consequence of this phosphorylation on PC synthesis is
this phosphorylation results in a stimulation of choline kinase
Inc. and acrylamide solutions were purchased from National Diagnostics.
and immunochemical reagents were purchased from Bio-Rad. Protein
chased from Pierce. Protein assay reagents, electrophoretic reagents,
plates and cellulose thin layer glass plates were from EM Science. DE52
heart) was purchased from Promega. Phospholipids were purchased
midine, aprotinin, leupeptin, pepstatin, polyvinylpyrrolidone, standard
Phenylmethylsulfonyl fluoride, bovine serum albumin, histone, benza-
sequencing kit was from Applied Biosystems. The Yeastmaker yeast
esis kit was purchased from Stratagene. The Prism DyeDeoxy DNA
plies were from Difco. Restriction enzymes, modifying enzymes, and
mids with the correct mutation. A third mutagenesis reaction was
mids YEp351 and pRS416 were digested with
and phosphatase (10 m M NaF, 5 m M
precleared by incubation with 0.15 ml of protein A-Sepharose CL-4B
fig. 1.  Pathways for the synthesis and turnover of PC in
S. cerevisiae. The pathways shown for the synthesis and turnover of PC
BEFORE FIG.4
Strains, Plasmas, and Growth Conditions—The strains and plas-
muts used in this work are listed in Table I. Methods for growth and
analysis of yeast were performed as described previously (32, 33). Yeast
cultures were grown in complete synthetic medium minus inositol (34),
containing 2% glucose and 30 µg/ml of choline at 30°C. Cells were in-
icubated with 100 µM (methyl-3H)choline (0.3 µCi/ml) and with 32P, (5
µCi/ml) for five to six generations to label CDP-choline pathway in-
mediates and phospholipids. For growth on plates, the media were supplemented with either 2% (yeast) and 1.5% (E. coli) uracil. Yeast cell numbers in liquid medium were deter-
microspectrophotometrically at an absorbance of 600 nm. The choline
excretion phenotype (35) was examined on complete synthetic medium plates
(minus inositol and choline) by using growth of a choline aux-
phosphorylates pure choline kinase on a serine residue, and
phosphorylation results in a stimulation of choline kinase activity
by a mechanism that increases catalytic turnover (31). The consequence of this phosphorylation on PC synthesis is
unknown and is the subject of this paper. Herein, we report the
identification of Ser30 and Ser85 as target sites of protein ki-
and phosphotidylylphosphatidylethanolamine; CDP-DAG, CDP-diacylglycerol; PA, phosphatidate.
tivity (17). ATP regulates the enzyme by promoting the oligo-
gomerization of the enzyme. ADP inhibits choline kinase activity
by a mechanism that affects the catalytic properties of the enzyme and the apparent affinity the enzyme has for the sub-
strates ATP and choline (17). Phosphorylation is another mechan-
ism by which yeast choline kinase is regulated (31). The enzyme is phosphorylated on multiple serine residues in vivo, and some of this phosphorylation is mediated by protein kinase A via the Ras-cAMP pathway (31). In vitro, protein kinase A phosphorylates pure choline kinase on a serine residue, and
this phosphorylation results in a stimulation of choline kinase activity
by a mechanism that increases catalytic turnover (31).
EXPERIMENTAL PROCEDURES
Materials—All chemicals were reagent grade. Growth medium sup-
plies were from Difco. Restriction enzymes, modifying enzymes, and
vent DNA polymerase were from New England Biolabs. Polymerase
chain reaction and sequencing primers were prepared commercially by
Genoys Biotechnologies, Inc. The QuikChange site-directed mutagen-
esis kit was purchased from Stratagene. The Prism DyeDeoxy DNA
sequencing kit was from Applied Biosystems. The Yeastmaker yeast
transformation system was from CLONTECH. The DNA size ladder
used for agarose gel electrophoresis was from Invitrogen. The plasmid
DNA purification and DNA gel extraction kits were from Qiagen, Inc.
Phenylnethysulfonfyl fluoride, bovine serum albumin, histone, benza-
midine, aprotonin, leupeptin, pepstatin, polyvinylpyrrolidone, standard
phosphoamino acids, choline, phosphocholine, and CDP-choline were
purchased from Sigma. The protein kinase A catalytic subunit (bovine
heart) was purchased from Promega. Phospholipids were purchased
from Avanti Polar Lipids. Silica Gel 60 thin layer chromatography plates
and cellulose thin layer glass plates were from EM Science. DE52
(DEAE-cellulose) was from Whatman. Radiochemicals were purchased
from PerkinElmer Life Sciences. Phosphocellulose filters were pur-
 chased from Pierce. Protein assay reagents, electrophoretic reagents,
and immunochemoical reagents were purchased from Bio-Rad. Protein
A-Sepharose CL-4B beads, polyvinylidene difluoride membrane, and
the enhanced chemiluminescence Western blotting detection kit were
purchased from Amersham Biosciences. Scintillation counting supplies
and acrylamide solutions were purchased from National Diagnostics.
Peptides were synthesized and purified commercially by Bio-Synthesis, Inc.

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containing 150 mM NaCl and 10 mM MgCl₂. 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 A phosphorylation. For in vivo labeling experiments, cell extracts were prepared with 50 mM Tris-HCl (pH 8.0) buffer containing protease and phosphatase inhibitors. Following immunoprecipitation, choline kinase proteins were dissociated from enzyme-antibody complexes (41), subjected to SDS-polyacrylamide gel electrophoresis (42), and transferred to polyvinylidene difluoride membranes (43). The 32P-labeled proteins were visualized and quantified by PhosphorImaging analysis.

For immunoblotting experiments, protein samples on polyvinylidene difluoride membranes were probed with a 1:5000 dilution of anti-choline kinase peptide antibodies. The choline kinase protein was detected using the ECF Western blotting chemiluminescent detection kit as described by the manufacturer. The choline kinase protein on immunoblots was acquired by FluorImaging analysis. The relative density of the protein was analyzed using ImageQuant software. Immunoblot signals were in the linear range of detectability.

Phosphorylation of Choline Kinase and Synthetic Peptides with Protein Kinase A—Immunoprecipitated choline kinase and choline kinase synthetic peptides were phosphorylated with protein kinase A using the bovine heart catalytic subunit. This enzyme is structurally and functionally similar to the S. cerevisiae protein kinase A catalytic subunit (44) and phosphorylates pure choline kinase under zero order kinetics (51). Phosphorylation reactions were measured for 10 min at 30 °C in a total volume of 40 μL. Reaction mixtures contained 50 mM Tris-HCl (pH 7.5), 60 mM dithiothreitol, 15 μM [γ-32P]ATP (4 μCi/μmole), 10 mM MgCl₂, protein kinase A, and immunoprecipitated choline kinase or synthetic peptides. For samples containing the immunoprecipitated choline kinase, the reaction was terminated by the addition of 1 mL of ice-cold radioimmune immunoprecipitation buffer. The protein A-Sepharose CL-4B beads were collected by centrifugation and washed three times with the same buffer. The beads were suspended in Laemml sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (42) followed by transfer to polyvinylidene difluoride membranes (43). The 32P-labeled proteins were visualized and quantified by PhosphorImaging analysis. For samples containing synthetic peptides, reactions were terminated by adding loading samples onto nitrocellulose filter paper. The filters were washed with 75 mM phosphoric acid and subjected to scintillation counting. Kinetic data for synthetic peptide substrates were analyzed according to the Michaelis-Menten equation using the EZ-FIT enzyme kinetic model-fitting program (45).

Tryptic Digestion and Two-dimensional Peptide Mapping—Polyvinylidene difluoride membrane slices containing 32P-labeled choline kinase or synthetic peptides were subjected to digestion with L-1-tosylamido-2-phenyl-ethyl chloromethyl ketone-trypsin and two-dimensional peptide mapping analysis as described by MacDonald and Kent (46). Electrophoresis (1% ammonium bicarbonate buffer at 1000 V for 20 min) and ascending chromatography (n-butyl alcohol/glacial acetic acid/pyridine/water, 10:3:12:15 for 7 h) were performed on cellulose thin layer glass plates. Dried plates were then subjected to PhosphorImaging analysis.

Preparation of Enzymes—For enzyme assays, cell extracts were prepared by disruption of yeast cells with glass beads using a Mini-BeadBeater-8 (BioSpec Products, Inc.) (47). The cell disruption buffer contained 50 mM Tris-HCl, 1 mM Na₃EDTA, 0.3 M sucrose, 10 mM 2-mercaptoethanol, and a protease inhibitor mixture. Glass beads and cell debris were removed by centrifugation at 1,500 × g for 5 min. The supernatant was used as the cell extract. Choline kinase was purified to homogeneity from Sf9 insect cells expressing the S. cerevisiae CKI1 gene as described by Kim et al. (17).

Choline Kinase Assay and Protein Determination—Choline kinase activity was measured for 45 min at 30 °C by following the formation of 3H-labeled phosphocholine from [methyl-3H]choline (2,000 cpm/nmol) as described previously (48). The reaction mixture contained 67 mM glycine-NaOH buffer (pH 9.5), 5 mM choline, 5 mM ATP, 10 mM MgSO₄, and enzyme protein in a final volume of 60 μL. Radiolabeled phosphocholine was separated from the radiolabeled substrate by the precipitation of the substrate as choline reineckate (48). The amount of labeled phosphocholine in the supernatant was determined by scintillation counting. The product phosphocholine was identified by thin layer chromatography on silica gel plates using the solvent system methanol, 0.5% sodium chloride, ammonium hydroxide (50:50:1, v/v) (49). The average S.D. of the enzyme assays (performed in triplicate) was ±3%. 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 method of Bradford (50) using bovine serum albumin as the standard.

Labeling and Analysis of CDP-choline Pathway Intermediates—The CDP-choline pathway intermediates phosphocholine and CDP-choline were labeled with [methyl-3H]choline (51, 52) and separated from whole cells after lipid extraction (53). The aqueous phase was neutralized, dried in vacuo, and the residue was dissolved in deionized water. Samples were subjected to centrifugation at 12,000 × g for 3 min to remove insoluble material. The CDP-choline pathway intermediates were separated by thin layer chromatography with silica gel 60 plates (49). The positions of the labeled intermediates on chromatograms were determined by PhosphorImaging analysis and compared with standards. The amount of labeled CDP-choline pathway intermediates was determined by liquid scintillation counting.

Labeling and Analysis of Phospholipids—Labeling of phospholipids with 32P, and with [methyl-3H]choline were performed as described previously (51, 54, 55). Phospholipids were extracted from labeled cells by the method of Bligh and Dyer (53) as described previously (56). Phospholipids were separated by DEAE-cellulose chromatography followed by one-dimensional thin layer chromatography on silica gel plates as described by Zhou et al. (57) with the modifications of Oshiro et al. (58). Elution of phospholipids from DEAE-cellulose (acetate form) was achieved with a step gradient of ammonium acetate (0, 80, 120 mM) in chloroform/methanol/water (2:3:1, v/v/v), and the solvent system chlorofom/pyridine/88% formic acid/methanol/water (60:35:10:5:2, v/v/v/v/v) was used for one-dimensional thin layer chromatography (58). The 32P-labeled phospholipids and 3H-labeled PC were visualized and...
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RESULTS

Choline Kinase Synthetic Peptides Containing a Protein Kinase A Sequence Motif Are Substrates for Protein Kinase A—Analysis of the deduced amino acid sequence of the CKI1 gene reveals that choline kinase has potential phosphorylation sites at Ser30 (S) and Ser85 (S) within the protein kinase A sequence motifs of RRHS and RRAS, respectively, at the N-terminal end of the protein (Fig. 2) (13). The peptides SQRRHSLTRQ (S30 peptide) and GPRRASATDV (S85 peptide), containing these two motifs, respectively, were synthesized based on the deduced sequence of choline kinase. We examined whether these peptides could serve as substrates for protein kinase A. Indeed, protein kinase A catalyzed the incorporation of the γ-phosphate of ATP into both peptides, and the dependence of activity on these substrates followed saturation kinetics (Fig. 3). The $V_{\text{max}}$ and $K_m$ values for the S30 peptide were 211 nmol/min/mg and 19.5 μM, respectively (Fig. 3A), and the $V_{\text{max}}$ and $K_m$ values for the S85 peptide were 58 nmol/min/mg and 377 μM, respectively (Fig. 3B). Based on kinetic constants, the S30 peptide was by far the better substrate for protein kinase A activity. The specificity constant ($V_{\text{max}}/K_m$) for the S30 peptide (10.8 μM$^{-1}$ min$^{-1}$ mg$^{-1}$) was 70-fold higher than that of the S85 peptide (0.15 μM$^{-1}$ nmol min$^{-1}$ mg$^{-1}$). The peptides SQRRHALTRQ and GPRRAAATDV were synthesized, where the serine residues within the RRHS and RRAS motifs were changed to alanine residues. These peptides were tested as substrates for protein kinase A phosphorylation. They did not serve as substrates for protein kinase A. These results indicated that the serine residues contained within the protein kinase A motifs of the S30 and S85 peptides were the target sites for phosphorylation.

Construction and Characterization of the CKI1-encoded Choline Kinase S30A, S85A, and S30A,S85A Mutants—Mutagenesis of Ser30 and Ser85 within the CKI1-encoded choline kinase was performed to examine the hypothesis that these sites are phosphorylated by protein kinase A. The codons for Ser30 and Ser85 were changed to alanine codons by site-directed mutagenesis. The mutations were made individually and in combination for the enzyme. The mutant and wild-type CKI1 alleles were expressed on a multicopy plasmid in a eki1Δ cki1Δ double mutant to obviate any effects due to the choline kinase activity expressed by the native EKI1 (59) and CKI1 (13) genes. A multicopy plasmid was used to increase expression of choline kinase to facilitate isolation of the phosphorylated forms of the enzyme from cell extracts. Cells bearing the mutant alleles of the CKI1 gene exhibited growth rates comparable with cells bearing the wild-type allele when grown vegetatively at 30 °C. In addition, no major morphological differences were observed in cells bearing the mutations.

The expression of the wild-type and mutant choline kinase proteins in exponentially growing cells was examined by immunoblot analysis. We used antibodies generated to a peptide sequence found at the N-terminal end of the deduced choline kinase protein that recognized pure enzyme (Fig. 4A). The analysis using these antibodies showed that the wild-type and mutant forms of choline kinase were expressed in cell extracts of the eki1Δ cki1Δ double mutant transformed with the plasmids bearing the CKI1 alleles (Fig. 4A). Like the purified choline kinase protein (17), the wild-type and mutant enzymes migrated on SDS-polyacrylamide gels with a subunit molecular mass of 73 kDa. Scanning densitometry showed that the levels of choline kinase protein on the immunoblots were essentially the same, indicating that the mutations did not affect the expression of the enzyme. We questioned what effect the mutations would have on the specific activity of choline kinase in cell extracts derived from cells bearing the CKI1 mutant alleles. The choline kinase activity found in the S30A and S85A mutants was reduced by 44 and 8%, respectively, when compared with the control (Fig. 4B). These results were consistent with the specificity of protein kinase A phosphorylation using the S30 and S85 peptides as substrates. The specific activity of choline kinase in cells bearing the combination S30A,S85A mutations was reduced by 60% relative to the control (Fig. 4B).
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Effect of the S30A, S85A, and S30A,S85A Mutations on the Phosphorylation of Choline Kinase in Vivo—We examined the effect of the S30A, S85A, and S30A,S85A mutations on the phosphorylation of the CKI1-encoded choline kinase in vivo. For these experiments, eki1Δ cki1Δ double mutant cells bearing plasmids with the wild-type and mutant alleles were labeled with $^{32}$P, followed by the immunoprecipitation of the choline kinase protein from cell extracts with anti-choline kinase antibodies. SDS-polyacrylamide gel electrophoresis of the immunoprecipitates, transfer to polyvinylidene difluoride membrane, and phosphorimaging analysis showed that the mutations caused a decrease in the extent of choline kinase phosphorylated in vivo (Fig. 5). The S30A, S85A, and S30A,S85A mutations caused a decrease in the extent of phosphorylation of choline kinase by 70, 17, and 83%, respectively. Immunoblot analysis showed that the wild-type and mutant choline kinase proteins were present at similar amounts.

Effect of the S30A, S85A, and S30A,S85A Mutations on the Phosphorylation of Choline Kinase by Protein Kinase A in Vitro—The effects of the S30A, S85A, and S30A,S85A mutations on protein kinase A phosphorylation of choline kinase were examined in vitro. For these experiments, the wild-type and mutant choline kinase proteins were immunoprecipitated from cell extracts and used as substrates. The immunoprecipitated proteins were incubated with protein kinase A and $^{32}$P-labeled ATP. Pure choline kinase was also phosphorylated with protein kinase A as a positive control. After the phosphorylation reactions, samples were subjected to SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and analyzed for radioactive label incorporated into choline kinase. The extent of phosphorylation of the S30A, S85A, and S30A,S85A mutant choline kinase proteins was reduced by 60, 13, and 96%, respectively, when compared with the wild-type enzyme (Fig. 6).

We examined the effects of the S30A and S85A mutations on the phosphopeptide map of the choline kinase. Choline kinase was immunoprecipitated from wild-type and S30A and S85A mutant cells. The immunoprecipitated proteins were phosphorylated with protein kinase A and $^{32}$P-labeled ATP, digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone trypsin, and subjected to two-dimensional phosphopeptide mapping analysis. Two major phosphopeptides, labeled 1 and 2 in Fig. 7A, were present in the peptide map of the wild-type choline kinase enzyme. The S30A mutation resulted in the loss of phosphopeptide 1, whereas the S85A mutation resulted in the...
loss of phosphopeptide 2 (Fig. 7A). These data indicated that Ser^{30} and Ser^{85} were contained in phosphopeptides 1 and 2, respectively. The phosphopeptide map of the S30A, S85A double mutant was not examined because the level of phosphorylation was too low for the analysis.

**Effect of the S30A, S85A, and S30A,S85A Mutations in Choline Kinase on the Incorporation of Choline into CDP-choline Pathway Intermediates**—Wild-type and S30A, S85A, and S30A,S85A mutant cells were labeled to steady state with 100 μM [methyl-^3H]choline to examine the effects of the mutations on the cellular concentrations of the CDP-choline pathway intermediates. A concentration of 100 μM was included in the growth medium to facilitate PC synthesis via the CDP-choline pathway (60), since, in the absence of choline, *S. cerevisiae* primarily synthesizes PC via the CDP-DAG pathway (4, 30, 61). The water-soluble fraction of exponential phase cells was analyzed for the CDP-choline pathway intermediates choline, phosphocholine, and CDP-choline by thin layer chromatography. Data for the incorporation of ^3H-labeled choline into the total pool of CDP-choline pathway intermediates are shown in Fig. 8A. The S30A, S85A, and S30A,S85A mutations caused a decrease in the total pool of intermediates by 55, 26, and 80%, respectively, when compared with wild-type cells (Fig. 8B). The effects of the mutations on cellular concentrations of choline and CDP-choline were less dramatic. The major effects were observed with the S30A,S85A double mutant, where the levels of choline and CDP-choline were reduced by 53 and 28%, respectively, when compared with wild-type cells (Fig. 8B).

**Effect of the S30A, S85A, and S30A,S85A Mutations in Choline Kinase on the Incorporation of Choline into PC and on Phospholipid Composition**—The effects of the S30A, S85A, and S30A,S85A mutations on PC synthesis via the CDP-choline pathway were examined by labeling cells to steady state with 100 μM [methyl-^3H]choline. Phospholipids were extracted from cells and analyzed by DEAE-cellulose chromatography followed by one-dimensional thin layer chromatography. The PC made via the CDP-choline pathway in the S30A, S85A, and S30A,S85A mutants was reduced by 58, 33, and 84%, respectively, when compared with wild-type cells (Fig. 9A). Cells were also labeled to steady state with ^32P, to examine the effects of the choline kinase mutations on overall phospholipid composition. ^32P, is incorporated into PC and other phospholipids by both the CDP-choline and CDP-DAG pathways (51, 52). Although the phosphorylation site mutations caused major decreases in PC made by the CDP-choline pathway, the mutations did not cause major effects on the overall PC content (Fig. 9B). With respect to the other major phospholipids, the S30A and S30A,S85A mutants exhibited a 30% increase in PE, and the S85A mutant exhibited a 50% decrease in PI. All three mutants exhibited an increase in phosphatidate (30–45%).
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**Effect of the S30A, S85A, and S30A,S85A Mutations on Choline Kinase on Phenotypes Associated with the sec14Δ cki1 Mutations**—The essential vesicular transport function of the SEC14-encoded PI/PC transfer protein (62, 63) can be suppressed (i.e., bypass) by mutations (e.g., cki1) in genes that encode CDP-choline pathway enzymes (64). Given the fact that the phosphorylation site mutations in choline kinase resulted in a decrease in choline kinase activity and a decrease in PC synthesis via the CDP-choline pathway, we questioned whether these mutations could bypass the sec14Δ lethal phenotype. Serial dilutions of sec14Δ cki1Δ mutant cells bearing the wild-type CKI1 gene and the CKI1S30A, CKI1S85A, and CKI1S30A,S85A mutant alleles on a single copy plasmid were inoculated onto agar plates and incubated at 25, 30, and 37 °C. As expected at the restrictive temperature (37 °C), the sec14Δ cki1Δ double mutant bearing empty plasmid grew, whereas cells bearing plasmid with the wild-type CKI1 allele did not grow. The cells bearing plasmid with the S30A, S85A, and S30A,S85A mutations in choline kinase did not grow at the restrictive temperature, whereas the cells with the mutations grew normally at the permissive temperatures (25 and 30 °C). Thus, the phosphorylation site mutations in the CKI1 gene did not suppress the essential function of the SEC14-encoded PI/PC transfer protein.

Cells that carry mutations (e.g., cki1) in the CDP-choline pathway enzymes exhibit a choline excretion phenotype, which is intensified when a mutation is combined with a sec14Δ mutation (35, 65). We examined whether the phosphorylation site mutations in choline kinase would elicit a choline excretion phenotype in a sec14Δ background. sec14Δ cki1Δ cells bearing the wild-type and mutant CKI1 alleles on the single copy plasmid were patched onto agar plates lacking choline and grown for 2 days at 30 °C. A choline auxotrophic mutant cho2 opi3 tester strain was then inoculated onto the plates and incubated for an additional 3 days at 30 °C (35). As expected (35, 65), the sec14Δ cki1Δ mutant with empty plasmid exhibited choline excretion as scored by the growth of the cho2 opi3 tester strain. However, cells bearing the wild-type and phosphorylation site mutant choline kinase enzymes did not excrete choline (data not shown).

**DISCUSSION**

The regulation of the CDP-choline pathway for PC synthesis is important to overall lipid metabolism and cell physiology in *S. cerevisiae* and in higher eukaryotic organisms (8). Choline kinase should play a pivotal role in its regulation, since the enzyme catalyzes the committed step in the pathway (Fig. 1) (9). The CKI1-encoded choline kinase of *S. cerevisiae* is subject to phosphorylation (31), a major mechanism by which enzymes are regulated (66, 67). Protein kinase A phosphorylates choline kinase on a serine residue and stimulates its activity by increasing the catalytic property of the enzyme (31). Identification of the protein kinase A target sites in choline kinase was addressed to gain information about the physiological consequence of enzyme phosphorylation on PC synthesis via the CDP-choline pathway. A combination of biochemical and molecular approaches were used to identify the protein kinase A phosphorylation sites in choline kinase. We examined the hypothesis that amino acid residues Ser30 and Ser85 contained in a protein kinase A sequence motif in the choline kinase (Fig. 2) were target sites for phosphorylation. The S30 and S85 peptides, which contained a protein kinase A sequence motif at Ser30 (RRHS) and Ser85 (RRAS), respectively, were substrates for protein kinase A in *vitro*. Based on kinetic constants, the S30 peptide was a much better substrate. The corresponding peptides containing RRHA and RRAA motifs, respectively, did not serve as substrates for protein kinase A. These data provided confidence that protein kinase A target sequences existed within the S30 and S85 peptides and that Ser30 and Ser85 in the choline kinase enzyme may be targets for phosphorylation. Ser30→Ala and Ser85→Ala mutations in the choline kinase enzyme were then constructed and used to support our hypothesis. The S30A, S85A, and S30A,S85A mutations did not affect the expression of choline kinase in cki1Δ cki1Δ double mutant cells but did cause a reduction in enzyme specific activity. The major effect on choline kinase activity was due to the S30A mutation alone and in combination with the S85A mutation. The S85A mutation had a much smaller effect on choline kinase activity. To further confirm that Ser30 and Ser85 were protein kinase A phosphorylation sites, the S30A, S85A, and S30A,S85A mutant choline kinase enzymes were isolated by immunoprecipitation and used as substrates for protein kinase A in *vitro*. Although the mutant enzymes were phosphorylated, the extent of their phosphorylation was reduced when compared with the wild-type enzyme. The S30A mutation alone and in combination with the S85A mutation caused the greatest reductions in protein kinase A phosphorylation (60 and 96%, respectively). Peptide mapping analysis of protein kinase A-phosphorylated choline kinase proteins showed that phosphopeptides 1 and 2 (Fig. 7) present in the wild-type enzyme were absent from the S30A and S85A mutant proteins, respectively. These data confirmed that Ser30 and Ser85 are specific targets for protein kinase A phosphorylation.

The S30A and S85A mutations resulted in a decrease in the phosphorylation of the choline kinase protein in *vitro*. The phosphorylation state of the protein correlated in general with the specific activity of choline kinase expressed in the wild-type and mutant enzymes. These data were consistent with the stimulatory effect that protein kinase A phosphorylation has on the activity of pure choline kinase (31). We addressed the...
physiological relevance of the phosphorylation of choline kinase on Ser\textsuperscript{30} and Ser\textsuperscript{85} by labeling mutant cells with [methyl-\textsuperscript{3}H]choline and following its incorporation into CDP-choline pathway intermediates and into PC. The effects of the S30A, S85A, and S30A,S85A mutations on the intermediate pool were manifested by reductions in the steady state levels of phosphocholine. Moreover, these mutations caused reductions in the steady state levels of PC labeled with [methyl-\textsuperscript{3}H]choline. These data were consistent with the decreased utilization of the CDP-choline pathway for PC synthesis in the mutants.

Data indicated that Ser\textsuperscript{30} was the major site for protein kinase A phosphorylation in vitro and in vivo. This was reflected by the negative effects the S30A mutation had on protein kinase A phosphorylation of immunoprecipitated choline kinase and on the cellular levels of the phosphorylated form of choline kinase, phosphocholine, and PC. Studies with the S85A mutation indicated that Ser\textsuperscript{85} by itself was less important. However, the near additive effects of the S30A and S85A mutations showed that phosphorylation of both Ser\textsuperscript{30} and Ser\textsuperscript{85} were important in the regulation of choline kinase activity and PC synthesis by the CDP-choline pathway.

Although the S30A, S85A, and S30A,S85A mutations in choline kinase caused decreases in the amount of PC synthesized by the CDP-choline pathway, the overall PC content of the mutants was not greatly affected. This was not surprising, since S. cerevisiae synthesizes PC by both the CDP-choline and CDP-DAG pathways (3, 4, 30). The mutants exhibited changes in the content of other phospholipids (e.g. PE, PI, and phosphatidate), but we can only speculate that CDP-DAG pathway enzymes may have been regulated to compensate for the defects in choline kinase phosphorylation. One candidate enzyme is PS synthase. This enzyme catalyzes the committed step in the CDP-DAG pathway (Fig. 1) and is one of the most highly regulated enzymes in phospholipid metabolism (3, 4, 30, 68). Protein kinase A phosphorylation is one of the ways PS synthase is regulated (69). Phosphorylation of PS synthase results in enzyme inhibition, which results in a decrease in PS synthesis (69, 70). Thus, a decrease in the phosphorylation of PS synthase would favor PC synthesis by PE methylation via the CDP-DAG pathway and compensate for the decrease in PC synthesis by the CDP-choline pathway. Another enzyme that may play a role in this regulation is phospholipase D. A decrease in phospholipase D activity and PC turnover could compensate for the decreased synthesis of PC by the CDP-choline pathway. Additional studies will be necessary to address these hypotheses along with other possible mechanisms that may be responsible for the regulation that occurs in cells with the phosphorylation site mutations.

Because the choline kinase phosphorylation site mutations caused a decrease in PC synthesis by the CDP-choline pathway, we questioned whether these mutations would elicit phenotypes associated with a sec14\textsuperscript{4} cki1Δ mutation (35, 64). However, the phosphorylation site mutations did not suppress the sec14\textsuperscript{4} lethal phenotype or elicit a choline excretion phenotype. Failure to suppress the sec14\textsuperscript{4} lethal phenotype may be explained by the fact that the choline kinase mutations did not obviate PC synthesis by the CDP-choline pathway. The choline excretion phenotype depends on activation of the spo14\textsuperscript{1} encoded phospholipase D-mediated turnover of PC synthesized by the CDP-DAG pathway and the inability of cells to reincorporate free choline into PC through the CDP-choline pathway (35, 65). Likewise, the suppression of the sec14\textsuperscript{4} lethal phenotype by CDP-choline pathway mutations is dependent on phospholipase D-mediated turnover of PC (65, 71). The requisite phospholipase D required for the sec14\textsuperscript{4} cki1Δ phenotypes may not be active in cells bearing the choline kinase phosphorylation site mutations. An alternative explanation for the lack of suppression of the sec14\textsuperscript{4} lethal phenotype by the choline kinase phosphorylation site mutations is that the overall PC content in the mutants was not significantly altered. This is consistent with recent work indicating that the overall PC content, rather than PC specifically synthesized by the CDP-choline or CDP-DAG pathways, is important for Golgi secretory function (72).

Choline kinase activity in S. cerevisiae (31) and in mammalian cells (73) is stimulated in response to Ras protein activation. The Ras-mediated signal transduction pathway in mammalian cells differs from that of S. cerevisiae. Protein kinase A is the principle mediator of signals transmitted through Ras proteins in yeast (74–76), whereas other protein kinases (e.g. Raf-1 kinase, protein kinase C, mitogen-activated protein kinase, mitogen-activated protein kinase kinase) are responsible for transmitting signals through Ras proteins in mammalian cells (77). Whereas yeast choline kinase activity is stimulated by protein kinase A phosphorylation (31), it is unknown whether mammalian forms of the enzyme are phosphorylated. The mechanism by which the mammalian form of choline kinase is stimulated in response to Ras protein activation is unclear (73).

In summary, the collection of data reported here supported the identification of Ser\textsuperscript{30} and Ser\textsuperscript{85} as protein kinase A target sites in the CKII-encoded choline kinase of S. cerevisiae. The phosphorylation of Ser\textsuperscript{30} alone and in combination with Ser\textsuperscript{85} was responsible for the major regulation of choline kinase activity and the synthesis of PC by the CDP-choline pathway. The availability of the phosphorylation site mutants will permit further studies on the regulation of phospholipid synthesis and the mechanism by which the Ras-cAMP pathway mediates cell growth.

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