Phosphorylation of *Saccharomyces cerevisiae* CTP Synthetase at Ser\(^{124}\) by Protein Kinases A and C Regulates Phosphatidylcholine Synthesis by the CDP-choline Pathway*

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CTP synthetase is an essential enzyme in all organisms. The essential nature of this enzyme emanates from the fact that the product of its reaction CTP is required for the synthesis of nucleic acids and membrane phospholipids (1). The enzyme catalyzes the ATP-dependent transfer of the amide nitrogen of glutamine to the C-4 position of UTP to form CTP (2, 3). GTP stimulates the reaction by accelerating the formation of a covalent glutaminyl enzyme catalytic intermediate (3–6). In eukaryotic cells, regulation of CTP synthetase activity plays an important role in the balance of nucleotide pools (6–12) and in the synthesis of membrane phospholipids (12–14). The importance of understanding the regulation of CTP synthetase is further emphasized by the fact that unregulated levels of CTP synthetase activity is a common property of various human cancers (15–22).

We utilize the yeast *Saccharomyces cerevisiae* as a model eukaryote to study the regulation of CTP synthetase and the impact of this regulation on phospholipid synthesis (Fig. 1). In yeast, CTP synthetase is encoded by the *URA7* (10) and *URA8* (11) genes. The yeast CTP synthetases (10, 11) contain a conserved glutamine amide transfer domain common to CTP synthetases from other organisms (24–33). The *URA7*-encoded CTP synthetase is more abundant than the *URA8*-encoded enzyme (34) and is responsible for the majority of the CTP synthesized *in vivo* (11). Like CTP synthetase from mammalian cells (35), the yeast enzymes are allosterically regulated by their substrates and product CTP (6, 34).

The *S. cerevisiae* *URA7*-encoded CTP synthetase is also regulated by phosphorylation. *In vivo*, CTP synthetase is phosphorylated on multiple serine residues (36). *In vitro* studies have shown that CTP synthetase is a substrate for protein kinase A (37) and for protein kinase C (36, 38). In *S. cerevisiae*, protein kinase A is the principal mediator of signals transmitted through the Ras-cAMP pathway (39, 40) whereas protein kinase C is required for the cell cycle (41–45) and plays a role in maintaining cell wall integrity (46). Independently, the phosphorylation of CTP synthetase by protein kinase A (37) and by protein kinase C (36, 38) results in the stimulation of CTP synthetase activity by a mechanism that increases catalytic turnover and decreases enzyme sensitivity to CTP product inhibition.

In this work, we addressed the question of whether the phosphorylation of CTP synthetase by protein kinase A affects the phosphorylation by protein kinase C. Amino acid residue Ser\(^{124}\) has been identified as the target site for protein kinase A phosphorylation in CTP synthetase (47). Therefore, we utilized a Ser\(^{124}\)A mutant CTP synthetase enzyme for our studies. The Ser\(^{124}\)A mutant enzyme is not phosphorylated in response to the activation of protein kinase A *in vivo*, and the mutant enzyme is not phosphorylated and stimulated by protein kinase A *in vitro* (47). This mutant enzyme exhibits lower catalytic activity and greater sensitivity to CTP product inhibition when compared with the wild type enzyme (47). These properties are consistent with the effects that protein kinase A phosphorylation has on the activity of wild type CTP synthetase (37, 47). We showed here that the Ser\(^{124}\)A mutation reduced the ability of CTP synthetase to be a substrate for protein kinase C. An

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Phosphorylation of Yeast CTP Synthetase

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were reagent grade. Growth medium supplements were purchased from Difco Laboratories. Nucleotides, l-glutamine, phenylmethylsulfonyl fluoride, benzamidine, aprotinin, leupeptin, pepstatin, histone, casein, choline, phosphocholine, and bovine serum albumin were purchased from Sigma. PVDF paper containing 32P-labeled CTP synthetase were subjected to phosphorimaging analysis. The positions of the labeled lipids on chromatography plates were compared with standard phospholipids after exposure to iodine vapor. The amount of each labeled phospholipid was determined by liquid scintillation counting of the corresponding spots on the chromatograms.

**Analysis of CDP-choline Pathway Intermediates**—Labeling of the CDP-choline pathway intermediates with [methyl-3H]choline was performed as described by McDonough et al. (14). Choline, phosphocholine, and CDP-choline were isolated from whole cells following lipid extraction (57). The aqueous phase was neutralized and dried in vacuo. The intermediates were detected by fluorography using EN3HANCE and compared with CDP-choline standards. Liquid scintillation counting was used to quantify the amounts of the intermediates.

**Data Analyses**—Kinetic data were analyzed according to the Michaelis-Menten equation using the EazyFit enzyme kinetic model-fitting program (61). Statistical analyses were performed with SigmaPlot 5.0 software.

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1 The abbreviations used are: PC, phosphatidylcholine; PVDF, polyvinylidene difluoride.
Stimulation of CTP Synthetase by Protein Kinase C—The URA7-encoded CTP synthetase is phosphorylated by protein kinase A (37) and by protein kinase C (36, 38). Residue Ser{sup 424} in the enzyme has been identified as the protein kinase A target site (47). We questioned whether phosphorylation at Ser{sup 424} affects phosphorylation of the enzyme by protein kinase C. Accordingly, we analyzed the phosphorylation of the S424A mutant CTP synthetase by protein kinase C. Protein kinase C was incubated with [γ-32P]ATP and various concentrations of the purified S424A mutant and wild type CTP synthetase enzymes. After the phosphorylation reactions, samples were subjected to SDS-PAGE and transferred to PVDF paper, followed by phosphorimaging analysis. Protein kinase C activity was dependent on the concentration of both the wild type and mutant forms of CTP synthetase (Fig. 2). The S424A mutation caused a decrease in enzyme phosphorylation at each CTP synthetase concentration (Fig. 2). The apparent {K}_{m} value for the S424A mutant enzyme (50 μg/ml) was 20-fold higher than that of the wild type enzyme (2.5 μg/ml). The effect of the S424A mutation on the dependence of protein kinase C activity on ATP concentration was also examined (Fig. 3). The extent of CTP synthetase phosphorylation at each ATP concentration was reduced for the S424A mutant enzyme when compared with the wild type enzyme. The apparent {K}_{m} value for the mutant enzyme (25 μM) was 2-fold higher when compared with the wild type enzyme (12.5 μM). The phosphorylation reactions using the S424A mutant CTP synthetase as a substrate were performed for different time intervals and with various concentrations of protein kinase C. The phosphorylation of the S424A mutant CTP synthetase by protein kinase C was time-dependent (Fig. 4) and dose-dependent (Fig. 5), but the rate and extent of phosphorylation was reduced by about 50% when compared with the wild type enzyme.

The effect of the S424A mutation on the stimulation of CTP synthetase activity by protein kinase C was examined. The purified S424A mutant and wild type CTP synthetase enzymes were phosphorylated with various concentrations of protein kinase C for 10 min. Following the phosphorylation reactions, samples were assayed for CTP synthetase activity using sub-saturating concentrations of UTP and ATP. These assay conditions were used to accentuate the effect of phosphorylation on the stimulation of CTP synthetase activity (36, 38). As described previously (36, 38), protein kinase C phosphorylation of the wild type enzyme resulted in a dose-dependent stimulation of CTP synthetase activity (Fig. 6). The activity of the S424A mutant enzyme was also stimulated by protein kinase C phosphorylation; however, the extent of stimulation was much reduced (Fig. 6). At the highest protein kinase C concentration, the stimulation of CTP synthetase activity was reduced by 80%.

A CTP Synthetase Synthetic Peptide Containing Amino Acid Residue Ser{sup 424} Is a Substrate for Protein Kinase C—The CTP synthetase peptide SLGRKDS{sup 424}HSA, which contains amino acid residue Ser{sup 424}, was synthesized based on the protein sequence of CTP synthetase. This synthetic peptide has been shown to be a substrate for protein kinase A (47). We examined whether this peptide could also serve as a substrate for protein kinase C. Protein kinase C catalyzed the phosphorylation of the...
peptide in a dose-dependent manner (Fig. 7). Analysis of the data yielded a $K_m$ value of 1 mM. The $K_m$ value for this peptide for protein kinase A phosphorylation is $30/\mu M$ (47). Thus, based on this assay, Ser$^{424}$ was a better target site for protein kinase A phosphorylation when compared with protein kinase C.

Effect of the S424A Mutation on the Phosphopeptide Map of CTP Synthetase Phosphorylated by Protein Kinase C—We examined the effect of the S424A mutation on the phosphorylation of CTP synthetase by protein kinase C using phosphopeptide mapping analysis. The purified S424A mutant CTP synthetase enzymes were phosphorylated with protein kinase C and $\gamma^{32}$P-labeled ATP followed by SDS-PAGE and transfer to PVDF paper. Papers containing the phosphorylated mutant and wild type enzymes were digested with L-1-tosyl-amido-2-phenylethyl chloromethyl ketone-trypsin and subjected to two-dimensional phosphopeptide mapping analysis. About equal amounts of the $32$P-labeled peptides derived from the wild type and mutant proteins were applied to the cellulose plates for comparison of the phosphopeptide maps. Five major phosphopeptides (labeled 1 through 5) were present in the phosphopeptide map of the wild type CTP synthetase enzyme (Fig. 8A). Quantification of the $32$P label showed that phosphopeptides 1 through 5 accounted for 26, 17, 24, 23, and 10%, respectively, of the $32$P-label incorporated into the wild type enzyme. For the S424A mutant CTP synthetase, phosphopeptides 1 through 4 were detected like the wild type enzyme, but
Cells bearing the wild type and S424A mutant CTP synthetase enzymes expressed from the single copy plasmids were grown in complete synthetic medium without inositol and choline to exclude regulatory effects that these precursors have on phospholipid synthesis (62, 64, 66). As described previously (47), immunoblot analysis showed that the S424A mutation did not affect the levels of the CTP synthetase protein. When grown in the absence of choline, wild type cells synthesize PC by both the CDP-diacylglycerol-dependent and CDP-choline-dependent pathways (12, 14, 67–69). The choline required for the CDP-choline pathway is derived from the phospholipase δ-mediated turnover of PC that is synthesized by way of the CDP-diacylglycerol pathway (69, 70). The composition of phospholipids was examined by labeling cells to steady state with both $^{32}P$ and $[^{3}H]$choline. $^{32}P$ is incorporated into phospholipids that are synthesized by way of the CDP-diacylglycerol-dependent and CDP-choline-dependent pathways (12, 14). Labeled choline is only incorporated into PC that is synthesized by way of the CDP-choline-dependent pathway (12, 14, 71). The concentration of choline added to the growth medium from the radioactive label was 0.1 $\mu$M, a concentration too low to affect the rate of synthesis of PC by the CDP-choline pathway (71).

The major effect of the S424A mutation on phospholipid composition was a 24% decrease for PC (Fig. 9A). This was PC synthesized by both the CDP-diacylglycerol-dependent and CDP-choline-dependent pathways. The amounts of the other major membrane phospholipids were not significantly affected by the S424A mutation (Fig. 9A). As indicated in Fig. 9B, $[^{3}H]$choline was incorporated into PC by way of the CDP-choline pathway (12, 14). Plotting the data as the ratio of the cpm of $[^{3}H]$choline incorporated into PC to the cpm of $^{32}P$, incorporated into PC was used to determine whether the S424A mutation affected the pathways by which cells synthesized PC (12, 14). Cells carrying the S424A mutation in CTP synthetase exhibited a 46% decrease in the ratio of $^{3}H$/$^{32}P$ incorporated into PC when compared with the control cells (Fig. 9B). These data indicated that the S424A mutation caused a decrease in the utilization of the CDP-choline-dependent pathway for PC synthesis.

The intermediates for the synthesis of PC by the CDP-choline-dependent pathway are phosphocholine and CDP-choline (72) (Fig. 1). The effect of the S424A mutation on the composition of these intermediates was examined by labeling cells with $[^{3}H]$choline. The amounts of phosphocholine and CDP-choline were reduced by 48 and 32%, respectively, in cells carrying the S424A mutation in CTP synthetase when compared with cells carrying the wild type enzyme (Fig. 10). The reduction in these intermediates was consistent with the conclusion that the S424A mutation caused a decrease in utilization of the CDP-choline pathway for PC synthesis.

**DISCUSSION**

CTP synthetase is an essential enzyme in *S. cerevisiae* (10, 11), because it provides the CTP that is required for the synthesis of nucleic acids and membrane phospholipids (1). Proper regulation of CTP synthetase in human cells is underscored by the fact that a number of cancers are characterized by unregulated levels of CTP synthetase activity (15–22). Covalent modification by phosphorylation is a major mechanism by which the activity of an enzyme may be regulated (73, 74). Previous studies have shown that the *S. cerevisiae URA7*-encoded CTP synthetase is phosphorylated by protein kinase A (37) and by protein kinase C (36, 38). These protein kinases have a major impact on the growth and metabolism of yeast (39, 41–46). The phosphorylation of CTP synthetase by protein kinases A and C results in the stimulation of CTP synthetase activity. Interest-
The S424A mutation resulted in about a 50% reduction in the phosphorylation of CTP synthetase by protein kinase C and an 80% reduction in the stimulatory effect that protein kinase C had on CTP synthetase activity. These effects could be attributed to a decrease in the rate of phosphorylation. In addition, the S424A mutation caused increases in the apparent $K_m$ values of CTP synthetase and ATP of 20- and 2-fold, respectively, in the protein kinase C reaction. Because protein kinase C phosphorylated CTP synthetase on multiple sites, the meaning of these effects is not straightforward. The decrease in the

ability of the S424A mutant CTP synthetase to be phosphorylated by protein kinase C was not because of any major effects on the overall structure of the enzyme. The S424A mutation does not affect the behavior of CTP synthetase during purification, the nucleotide-dependent tetramerization of the purified enzyme, or the stability of the enzyme to temperature (47).

An explanation for the decreased ability of the S424A mutant enzyme to be phosphorylated by protein kinase C was that Ser$^{424}$ was a target site for both protein kinase A and protein kinase C. Indeed, the phosphopeptide that was present in the phosphopeptide map of the protein kinase C-phosphorylated wild type enzyme but absent from the map of the protein kinase C-phosphorylated S424A mutant enzyme was the same phosphopeptide derived from the wild type enzyme phosphorylated by protein kinase A. Phosphorylation of Ser$^{424}$ accounted for 10% of the total phosphorylation of CTP synthetase by protein kinase C. In addition, the CTP synthetase synthetic peptide containing Ser$^{424}$ that is a substrate for protein kinase A (47) was also a substrate for protein kinase C. That Ser$^{424}$ was phosphorylated by protein kinases A and C provides a plausible explanation why the combined effect of these phosphorylations on CTP synthetase activity is small (37). Overall, the data support the hypothesis that phosphorylation of Ser$^{424}$ by protein kinase A (or by protein kinase C) was required for maximum phosphorylation and stimulation of the enzyme by protein kinase C.

We addressed the physiological relevance of the phosphorylation of Ser$^{424}$ with respect to phospholipid synthesis using cells carrying the S424A mutant CTP synthetase. The in vivo labeling experiments showed that the S424A mutation resulted in a decrease for PC. The decrease in PC content could be attributed to a decrease in the utilization of the CDP-choline pathway, because the ratio of the cpm of [methyl-$^3$H]choline incorporated into PC to the cpm of $^{32}$P incorporated into PC was reduced in cells with the S424A mutant enzyme. This conclusion was further supported by the reduced amounts of phosphocholine and CDP-choline in cells with the S424A mutant enzyme.
The reason for the decrease in phosphocholine was unclear. One would expect that the level of phosphocholine would accumulate, because CTP is the direct precursor of CDP-choline in the response (Fig. 1). The reduction for phosphocholine may be attributed to the down-regulation of the choline kinase enzyme in response to the mutation in CTP synthase. The reduced amount of PC in cells with the S424A mutation did not appear to be from a reduction in utilization of the CDP-diacylglycerol-dependent pathway. The amounts of the phospholipids (e.g., phosphatidylserine and phosphatidylethanolamine) in this pathway were not significantly affected by the S424A mutation. Thus, the phosphorylation of CTP synthase at Ser424, whether it is mediated by protein kinase A and/or by protein kinase C, plays a role in the regulation of PC synthesis via the CDP-choline pathway. Differentiating between which protein kinase phosphorylates Ser424 and regulates phospholipid synthesis in vivo will require additional studies.

Protein kinase C phosphorylated CTP synthase at sites other than Ser424. Recent studies have shown that Ser36, Ser330, Ser354, and Ser454 are also target sites for protein kinase C phosphorylation (23). Whereas Ser424 was phosphorylated by protein kinases A and C, Ser36, Ser330, Ser354, and Ser454 are not phosphorylated by protein kinase A. Protein kinase A only phosphorylates Ser424 (47). Phosphorylation of CTP synthase by protein kinase C is not straightforward. For example, phosphorylation at Ser330 affects the phosphorylation of CTP synthase at other protein kinase C sites (23). Moreover, phosphorylation at the different protein kinase C target sites has different effects on CTP synthase activity (23). It is unknown whether the phosphorylation of CTP synthase at Ser36, Ser330, Ser354, and Ser454 by protein kinase C affects phosphorylation by protein kinase A. Studies are in progress to address this question. Clearly, the phosphorylation and regulation of CTP synthase is complex. This complex regulation is likely to represent a mechanism by which various signal transduction pathways mediate CTP synthase activity and its roles in cell physiology.

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