Regulation of Human Cytidine Triphosphate Synthetase 1 by Glycogen Synthase Kinase 3\(^*\)

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Cytidine triphosphate synthetase (CTPS) catalyzes the rate-limiting step in the \textit{de novo} synthesis of CTP, and both the yeast and human enzymes have been reported to be regulated by protein kinase A or protein kinase C phosphorylation. Here, we provide evidence that stimulation or inhibition of protein kinase A and protein kinase C does not alter the phosphorylation of endogenous human CTPS1 in human embryonic kidney 293 cells under the conditions tested. Unexpectedly, we found that low serum conditions increased phosphorylation of endogenous CTPS1 and this phosphorylation was inhibited by the glycogen synthase kinase 3 (GSK3) inhibitor indirubin-3′-monoxime and GSK3β short interfering RNAs, demonstrating the involvement of GSK3 in phosphorylation of endogenous human CTPS1. Separating tryptic peptides from \([32\text{P}]\)orthophosphate-labeled cells and analyzing the phosphopeptides by mass spectrometry identified Ser-571 and Ser-575 as phosphorylated residues. Mutation of Ser-571 demonstrated that Ser-571 was the major site phosphorylated by GSK3 in intact human embryonic kidney 293 cells by GSK3 \textit{in vitro}. Furthermore, mutation of Ser-575 prevented the phosphorylation of Ser-571, suggesting that phosphorylation of Ser-575 was necessary for priming the GSK3 phosphorylation of Ser-571. Low serum was found to decrease CTPS1 activity, and incubation with the GSK3 inhibitor indirubin-3′-monoxime protected against this decrease in activity. Incubation with an alkaline phosphatase increased CTPS1 activity, and incubation with the GSK3 inhibitor indirubin-3′-monoxime protected against this decrease in activity. CTPS activity is regulated, primarily in bacteria and yeast, has uncovered two major forms of regulation, regulation by metabolites and phosphorylation. The unique ability of CTPS to bind four nucleotides (UTP, ATP, GTP, and CTP) and the amino acid glutamine makes CTPS sensitive to the metabolite levels of four nucleotides and glutamine in cells, providing exquisite metabolic control of CTPS.

Pyrimidine nucleotides have been known for decades to be essential components of cells. Most recently the effects of nucleotides have been revisited and shown to regulate apoptosis some formation by binding to cytochrome c, and constitutively high levels of nucleotides were shown to inhibit the cell cycle and the response to DNA damage (1, 2). Of all the nucleotides, the cellular concentration of cytidine triphosphate (CTP) is the lowest, suggesting that control of CTP synthesis is tightly regulated (3). CTP is important in DNA/RNA synthesis, phospholipid synthesis, and protein sialylation, which are needed for cell proliferation and cell size expansion. CTP levels are elevated during S phase of the cell cycle, and rapidly proliferating cells maintain a higher basal concentration of CTP and dCTP (4–10). Cytidine nucleotide levels were also found to be elevated in a variety of tumors and elevated, albeit to a lesser degree, in normal proliferating cells (11, 12). Primary human lymphocytes demonstrate a dramatic increase in CTP levels following mitogenic stimulation, demonstrating the importance of CTP synthesis in response to mitogens (12).

Cytidine triphosphate synthetase (CTPS\(^2\); EC 6.3.4.2) catalyzes the rate-limiting step in the \textit{de novo} formation of CTP; in mammalian cells CTPS catalyzes the amination of UTP to form CTP, with glutamine serving as the amine donor and ATP being hydrolyzed during the process. CTPS activity is feedback-inhibited by CTP, and with glutamine as a substrate CTPS activity is markedly enhanced by GTP. In mammals there are two CTPS isozymes that share 74% amino acid identity (13). In the yeast \textit{Saccharomyces cerevisiae} there are also two isoforms of CTPS, URA7 and URA8, corresponding to the mammalian enzymes CTPS1 and CTPS2, respectively. Investigation into how CTPS is regulated, primarily in bacteria and yeast, has uncovered two major forms of regulation, regulation by metabolites and phosphorylation. The unique ability of CTPS to bind four nucleotides (UTP, ATP, GTP, and CTP) and the amino acid glutamine makes CTPS sensitive to the metabolite levels of four nucleotides and glutamine in cells, providing exquisite metabolic control of CTPS.

Regulation of yeast CTPS by phosphorylation was discovered by Carman and co-workers (14), who showed that yeast CTPS1 (URA7) was phosphorylated \textit{in vitro} on multiple sites by protein kinase C (Ser-36, Ser-330, Ser-354, and Ser-454). Mutation of potential phosphorylation sites demonstrated that Ser-330 was a major site of protein kinase C phosphorylation (14). Additionally, this same group identified Ser-424 as an \textit{in vitro} protein kinase A phosphorylation site (15). The S424A mutant showed decreased CTPS activity and greater sensitivity to CTP feedback inhibition (15). Recently this same group also showed

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\(^2\) The abbreviations used are: CTPS1, CTP synthetase 1; HEK, human embryonic kidney; GSK3, glycogen synthase kinase 3; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; RIPA, radioimmuno precipitation buffer; ddH2O, double distilled H2O; CIP, calf intestinal phosphatase; siRNA, small interfering RNA; WT, wild type; ERK, extracellular signal-regulated kinase; TPCK, i-1-tosylamido-2-phenylethyl chloromethyl ketone.
that human CTPS1 expressed in yeast was phosphorylated by protein kinase A (16). However, sequence alignments of the yeast CTPS1 phosphorylation sites with mouse and human CTPS1 indicate that only two of the phosphorylation sites identified in yeast CTPS1 (URA7) are conserved in humans (supplementary Fig. S1), suggesting that human CTPS1 in human cells may be phosphorylated on different sites than URA7.

Despite the importance of CTP in processes such as phospholipid synthesis and protein sialylation, little is known about the regulation of human CTPS1. The objective of this study was to investigate the phosphorylation and regulation of mammalian CTPS1 expressed in mammalian cells. Endogenous human CTPS1 was found to be phosphorylated by glycogen synthase kinase 3 (GSK3) in response to low serum, and several phosphorylation sites in human CTPS1 were mapped to the carboxyl terminus, including the site for GSK3 phosphorylation. Phosphorylation of endogenous human CTPS1 was found to inhibit CTPS1 activity, and inhibition of GSK3 restored the low serum decrease in CTPS1 activity. Thus, these studies suggest that human CTPS1 expressed in human cells is regulated by GSK3 phosphorylation of CTPS1, a carboxyl terminal residue not found in the yeast enzyme.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human embryonic kidney (HEK) 293 cells purchased from the University of North Carolina Tissue Culture facility were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (high glucose) supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS) in an atmosphere of 5% CO₂.

**Plasmid Construction and Site-directed Mutagenesis**—Full-length cDNA containing human CTPS1 was purchased from Invitrogen (IMAGE clone ID 3355881). A carboxyl-terminal FLAG sequence was introduced after insertion of human CTPS1 into pCDNA 4 myc/his using the compatible cohesive ends EagI & NotI and XhoI. Briefly, the CTPS1 IMAGE clone was digested with EagI and XhoI and ligated into pCDNA4 digested with NotI and XhoI. Introduction of the carboxyl-terminal FLAG sequence was done by first amplifying a carboxyl-terminal fragment of CTPS1 with the sense primer 5′-CGCGCCGCCACATGAAAGTACATTCTG-3′ (HindIII site is underlined) and antisense primer 5′-GACTCGAGGGCTTGTGCATCGTCTTGTGTAATCCGGCGCATGATTTATGGAGG-3′ (XhoI site is underlined, FLAG sequence (DYKDDDDK) is boldface, and mutation of stop codon to alanine is italicized and underlined) and finally ligating the carboxyl-terminal-amplified section back into the pCDNA4 CTPS1 using HindIII and XhoI, digesting the amplified amino-terminal fragment with NotI and EcoN1, and ligating the digested amino-terminal fragment into pCDNA4 myc/his CTPS1-FLAG digested with NotI and Eco N1. Site-directed mutagenesis was done using QuikChange™ (Stratagene) according to the manufacturer’s instructions. All mutations were verified by sequencing. Of note, Ser-571 was found to be an isoleucine in the IMAGE clone and was mutated back to the wild type serine.

**Transfection and Immunoprecipitation of Endogenous and Recombinant CTPS1**—HEK 293 cells were transfected using Lipofectamine™ (Invitrogen) as instructed by the manufacturer. Anti-FLAG (Sigma) resin was prepared as recommended by the manufacturer to remove non-covalently linked antibodies. Approximately 8 × 10⁶ cells were washed twice with ice-cold PBS and lysed with 0.5 ml of radioimmune precipitation (RIPA) buffer without SDS (150 mM NaCl, 9.1 mM Na₂HPO₄, 1.7 mM NaH₂PO₄, 1% Nonidet P-40 substitute, and 0.5% sodium deoxycholate) containing 1 μg/ml leupeptin, 10 mM microcystin, 1 mM Na₂VO₄, and 250 μM phenylmethylsulfonyl fluoride. Following clarification of lysate by centrifugation, 1 mg of lysate was rotated with 10 μl of anti-FLAG resin overnight at 4 °C. Immunoprecipitation of endogenous CTPS1 was done by rotating 1 mg of lysate clarified by centrifugation with 10 μl of anti-CTPS1 antibody overnight at 4 °C. 10 μl of protein A-agarose beads (Santa Cruz Biotechnology) was added to lystate containing anti-CTPS1 antibody and rotated an additional hour at 4 °C.

**CTPS Activity Assay**—CTPS1 was immunoprecipitated as described above. Immunoprecipitates were washed three times with 0.5 ml of RIPA buffer without SDS containing 5 μg/ml leupeptin and a final wash was done with 1 ml of 1× CTPS reaction buffer (50 mM Tricine, pH 8.1, 20 mM MgCl₂, and 5 mM KCl). The volume was adjusted to 40 μl with 10 μl of 5× reaction buffer and 30 μl of double distilled H₂O (ddH₂O). CTPS1 and 5× substrate mix (5 mM ATP, 0.5 mM UTP, 5 mM GTP, 100 mM L-glutamine, and 0.625 μCi/ml [¹⁴C]UTP or 0.1 μCi/ml [³²P]UTP) were equilibrated to 37 °C. The reaction was initiated by adding 10 μl of 5X substrate mix to 40 μl of CTPS1 in reaction buffer and stopped after 45 min by adding 10 μl of 200 mM EDTA and removing the tube to ice. CTPS1 reactions were reduced to dryness using a SpeedVac. Dried supernatant pellets were resuspended in 10 μl of ddH₂O. 3–4 μl of resuspended pellets and 1 μl of 100 mM UTP/CTP/UDP/CDP standards were spotted onto polyethyleneimine cellulose plastic-backed plates (Fisher Scientific) that were prewashed for 10 min with ddH₂O, 5 min with 0.65 N HCl, and 1 min with methanol. Nucleotides were resolved using 0.65 N HCl as the ascending chromatography buffer. Areas corresponding to UTP, CTP, UDP, and CDP were visualized by exposing TLC plate to a phosphorimaging screen overnight. Areas corresponding to UTP, CTP, UDP, and CDP were excised and counted using Ecoscint™ (Fisher Scientific) scintillation fluid.

**Antibodies and Immunoblotting**—Anti-CTPS1 (human) antibody was generated by Rockland Immunochemicals using a carboxyl-terminal peptide (amino acids 578–591, H₂N-CSEIKLKFPSINHD-COOH) containing an amino-terminal cysteine for coupling to peptide affinity column and keyhole limpet hemocyanin. Anti-CTPS1 antibody was purified from rabbit serum in a three-step process. First, serum proteins including immunoglobulins were precipitated at 4 °C by adding ice-cold saturated (NH₄)₂SO₄ to a final percentage of 50%.
overnight at 4 °C against PBS. Second, 2.5 ml of (NH₄)₂SO₄ at a volume of PBS equal to the initial volume of serum and dialyzed overnight against PBS. Washing the protein A column with 100 mM Tris, pH 8.0. After washing the protein A column with 100 mM Tris, pH 8.0, bound antibodies were eluted with 5 ml of 50 mM glycine, pH 3.0. 1-mI fractions were collected in Eppendorf tubes containing 100 μl of 1 × Tris, pH 8.0, to immediately neutralize pH. All fractions were pooled and dialyzed overnight at 4 °C against PBS. Lastly, antibodies specific for CTPS1 were separated from nonspecific antibodies using a peptide affinity column created with the antigenic peptide (H₂N-CSEITEKFPSINHD-COOH) and a Sulforlink column according to the manufacturer’s instructions. Eluted anti-CTPS1 antibodies were concentrated to −350 μl and buffer exchanged to PBS with 0.05% NaN₃ using 4 ml of 10-KD MWCO spin concentrators. 20-μl aliquots were stored at −20 °C for future use.

Immunoblotting for GSK3 and phospho-GSK3 (α/β; Ser-21/Ser-9) was done by lysing HEK 293 cells with RIPA without SDS and determining the protein concentration in lysate using the method of Bradford (17). Lysates were prepared for SDS-PAGE by adding an equal volume of 2 × SDS sample buffer (0.5 × Tris, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.002 g/ml bromphenol blue). 10–40 μg of protein was separated on an 8 or 10% discontinuous buffer gel, and proteins were transblotted to polyvinylidene fluoride membrane (Immobilin-P; Millipore). Membranes were blocked for 1 h at room temperature or overnight in a cold room with 3% w/v cold fish gelatin (Sigma) diluted with TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20). Primary antibodies (1:1000 α-phospho-(Ser-9) GSK3β (Cell Signaling) and 1:1000 α-GSK3β (Cell Signaling)) were diluted in 1% w/v gelatin in TBST and incubated with membranes for 1 h at room temperature (α-GSK3β) or overnight at 4 °C (α-phospho-(Ser-9) GSK3β; Cell Signaling). Membranes were then washed with TBST three times for 5 min each wash and incubated for 1 h with 1:2000 goat anti-rabbit (Santa Cruz) conjugated to horseradish peroxidase (for α-GSK3β) or alkaline phosphatase (for α-phospho-(Ser-9) GSK3β). Membranes were washed three times for 5 min each wash with TBST and visualized using an enhanced chemiluminescent kit (Amersham Biosciences) according to the manufacturer’s instructions and exposing membrane to x-ray film or using a BCIP/NBT kit (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) (Promega) to visualize alkaline phosphatase-conjugated secondary antibody as suggested by the manufacturer.

GSK3β RNA Interference—3.5 × 10⁵ HEK 293 cells were plated onto 60-mm poly-D-lysine-coated plates and incubated overnight. DMEM containing 10% FBS and penicillin/streptomycin (DMEM + 10% FBS + pen./strept.) was removed from cells, and medium was replaced with DMEM containing 10% FBS and no antibiotics (DMEM + 10% FBS). RNA duplexes (Dharmacon) were suspended as suggested by the manufacturer, and 15 μl of 20 μm RNA duplex was added to one tube containing 288 μl of DMEM + 10% FBS and mixed thoroughly. Tubes containing RNA duplexes and Dharmafect 1 were combined, mixed thoroughly, and allowed to stand at room temperature for 20 min. 2400 μl of DMEM + 10% FBS was added to the duplex/Dharmafect 1 mixture to give a total volume of 3000 μl, and the entire mixture (3000 μl) was added to plates that had their medium removed. Medium was switched the following day to DMEM + 10% FBS + pen./strept. Cellular manipulations were done the following day after incubating cells overnight.

In Vitro GSK3β Kinase Reaction—Endogenous CTPS1 or FLAG-CTPS1 was immunoprecipitated as described before. Immunoprecipitations were washed one time with 1 ml of DMEM GSK3 reaction buffer (20 mM Tris, pH 7.5, at 25 °C and 10 mM MgCl₂) without dithiothreitol and 25 μl of complete GSK3 reaction buffer (20 mM Tris, pH 7.5, at 25 °C, 10 mM MgCl₂, 10 μM ATP, 2 μl of [γ⁻³²P]ATP (10 mCi/ml; PerkinElmer), 5 μg/ml leupeptin, 10 nM microcystin, and 5 mM dithiothreitol) was added to each immunoprecipitate. To each reaction 0.01 units of GSK3β-purified enzyme (New England Biolabs) were added and incubated at 30 °C for 30 min, flicking tubes occasionally to resuspend immunoprecipitates. Reactions were stopped by removing the supernatant, placing tubes on ice, and adding 30 μl of 1 × SDS sample buffer (see “Antibodies and Immunoblotting” for composition of SDS sample buffer) to immunoprecipitates.

Calf Intestinal Phosphatase Treatment of CTPS1—Immunoprecipitated CTPS1 (prepared as described earlier) was washed three times with 1 ml of RIPA buffer without SDS and one time with 1 ml of 1 × CTPS reaction buffer. 10 μl of 5 × CTPS reaction buffer and 25 μl of ddH₂O were added to immunoprecipitates, and tubes were placed at 30 °C for phosphate reaction. Dephosphorylation was initiated by adding 15 μl (1 unit/μl) of calf intestinal phosphatase (CIP; Roch Biochemicals) or 15 μl of 50% glycerol (control reaction) to tubes containing immunoprecipitated CTPS1 (50-μl total volume). Reactions were stopped by adding 1 ml of ice-cold 1 × CTPS reaction buffer to dilute reaction components and placing tubes on ice. Immunoprecipitates were washed again with ice-cold 1 × CTPS reaction buffer.

In Vivo Labeling with [³²P]Orthophosphate—100-μm tissue culture plates (Sarstedt Inc.) were coated with 0.02 mg/ml poly-D-lysine (Sigma Aldrich) and washed with ddH₂O. 7.5 × 10⁵ HEK 293 cells were plated on poly-D-lysine-coated plates. Cells were washed one time with 5 ml of phosphate-free DMEM (phosphate/pyruvate-free DMEM (Invitrogen) supplemented with sodium pyruvate (Invitrogen), giving a final concentration of 110 mg/liter sodium pyruvate and the same concentration of penicillin/streptomycin as described earlier). 3 ml of phosphate-free DMEM containing 10% dialyzed FBS (Invitrogen) was added to plates 30 min prior to adding 1 mCi of [³²P]orthophosphate/plate (0.33 mCi/ml; MP Biomedical). In a typical experiment, cells were labeled for 4 h prior to manipulation. Labeling was stopped by washing plates twice with 5 ml of ice-cold PBS. Cells were lysed by scraping plates in the presence of 0.5 ml of no-SDS RIPA buffer. Immunoprecipitations were done as described above. Immunocomplex proteins were eluted from beads by boiling for 3 min with Laemmli buffer. SDS-PAGE/autoradiography was done on eluted proteins.
Phosphorylation Site Identification by Mass Spectrometry—HEK 293 cells were labeled and immune complexes were separated as described above. After separation of eluted proteins by SDS-PAGE, proteins were transferred to nitrocellulose (Osmonics; GE Healthcare). Proteins were visualized using MemCode (Pierce Biotechnologies) according to the manufacturer’s instructions. Bands corresponding to CTPS1 were excised and cut into ~1-mm pieces. Membrane pieces were placed in a siliconized microfuge tube, and MemCode destaining was done as recommended by the manufacturer. After

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**A**

![Autoradiograph](image)

| % Serum | 10 | 0.1 |
|---------|----|-----|
| Autoradiograph | ![Image](image) |
| Total CTPS1 | ![Image](image) |

![Graph](image)

**B**

![Autoradiograph](image)

| % FBS | 10 | 0.1 |
|-------|----|-----|
| Autoradiograph (CTPS1) | ![Image](image) |
| Phospho-GSK3β (S9) | ![Image](image) |

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**C**

| 10% FBS | 0.1% FBS |
|---------|---------|
| 10 μM Ind. DMSO | + | + |
| Autoradiograph | ![Image](image) |
| Total CTPS1 | ![Image](image) |

| 0.1% Serum |
|------------|
| RNAi Target: Non-targeting GSK3β |
| Phosphoimage | ![Image](image) |
| Total CTPS1 | ![Image](image) |

![Graph](image)

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**D**

| 0.1% Serum |
|------------|
| RNAi Target: Non-targeting GSK3β |
| Phosphoimage | ![Image](image) |
| Total CTPS1 | ![Image](image) |

![Graph](image)
membrane pieces were washed three times with ddH2O, they were incubated with 1% w/v polyvinylpyrrolidone suspended in 100 mM acetic acid for at least 1 h at 37 °C. Membrane pieces were washed four times with ddH2O to ensure residual polyvinylpyrrolidone was removed. Membrane pieces were incubated with 0.5 μg of TPCK-modified sequencing grade trypsin (Promega) in 25 mM ammonium bicarbonate, pH 8.0, overnight at 37 °C. Peptides were collected by removing the trypsin solution to a clean tube, washing the membrane pieces three times with ddH2O, and removing each ddH2O wash to the same clean tube containing the trypsin solution. The volume of collected peptides was reduced to a final volume of ~10 μl using a speed vac. Recovery of radioactivity from membrane pieces was at least 85% as determined by Cerenkov counting. For separation of peptides on cellulose TLC plate, peptides were washed at least three times with ddH2O to remove ammonium bicarbonate. After the final ddH2O wash, peptides were resuspended in the first dimension buffer (pH 1.9 buffer, 15% v/v acetic acid, and 5% v/v of 88% formic acid) to give a final volume of ~8 μl. The peptides were spotted in a corner of a 10 × 10-cm cellulose TLC plate, 1.5 cm from the sides of the plate. Peptides were electrophoresed in a Hunter thin layer electrophoresis box at 1000 V for 32 min. After thoroughly drying the TLC plate, peptides were separated in the second dimension using Scheidtmann buffer (isobutyric acid-pyridine-acetic acid-butanol-water, 65:5:3:2:29) (18). After exposure of TLC plates to x-ray film or phosphorimaging plate, radioactive spots indicative of phosphopeptides were eluted as described previously (19). Volume of peptides was decreased to ~10 μl using a speed vac. Peptides were subjected to nano-electrospray ionization mass spectrometry on an Applied Biosystems QSTAR® pulsar mass spectrometer in positive ion mode. Peptides were sequenced by nano-electrospray ionization tandem mass spectrometry using BioAnalyst software.

Data Analysis—Statistical analysis was done using one-way analysis of variance with a Tukey post-hoc test and a 95% confidence interval.

RESULTS

CTPS1 Is Phosphorylated in Response to Serum Starvation—Inhibition or activation of protein kinase A or protein kinase C did not alter the phosphorylation of endogenous human CTPS1 from [32P]orthophosphate-labeled HEK 293 cells (supplemental data). Instead, we observed that CTPS1 phosphorylation increased after incubation overnight in low serum (0.1% FBS). The average increase in radioactivity associated with endogenous CTPS1 isolated from [32P]orthophosphate-labeled HEK 293 cells in 0.1% FBS was approximately twice that from cells maintained in 10% FBS. Two-dimensional thin layer chromatography separation of tryptic peptides from endogenous CTPS1 revealed five major phosphopeptide spots; consistent with the effects of low serum on CTPS phosphorylation, all five of these spots increased after serum starvation (Fig. 1A).

Involvement of GSK3 in Phosphorylation of Endogenous CTPS1—Because we observed that phosphorylation of CTPS1 increased in low serum (0.1% FBS) conditions, kinases known to be activated under these conditions were investigated. Two kinases shown to be activated under low serum/nutrient starvation conditions are AMP-activated protein kinase and GSK3 (20, 21). Incubation of HEK 293 cells with the AMP-activated protein kinase inhibitor compound C failed to inhibit the increase in CTPS1 phosphorylation induced by low serum (data not shown). Analysis of GSK3 demonstrated that the inhibitory phosphorylation site on GSK3 α/β (Ser-21, Ser-9 respectively) was decreased in 0.1% FBS, indicating that GSK3 was more active when HEK 293 cells were maintained overnight in low serum (Fig. 1B). To test whether GSK3 was involved in CTPS1 phosphorylation, HEK 293 cells were incubated with the GSK3 inhibitor indirubin-3’-monoxime (indirubin) or the vehicle (Me2SO) overnight in the presence of 10 or 0.1% FBS. The increase in low serum-induced CTPS1 phosphorylation was inhibited by indirubin, suggesting GSK3 was a candidate kinase necessary for the phosphorylation of CTPS1 (Fig. 1C). Inhibition of GSK3 prevented the low serum-induced phosphorylation of CTPS1 in four separate experiments. Shown is a representative experiment. Furthermore, two-dimensional phosphopeptide mapping showed that indirubin decreased the radioactivity of the five phosphopeptide spots equally (Fig. 1C).

To further establish that GSK3 was specifically involved in low serum-induced CTPS1 phosphorylation, GSK3β protein expression was specifically reduced by small interfering RNA (siRNA) (Fig. 1D). GSK3β siRNA almost completely eliminated GSK3β expression but did not affect GSK3α expression (Fig. 1D). Analysis of CTPS1 phosphorylation in these cells in two separate experiments demonstrated that low serum-induced CTPS1 phosphorylation was inhibited in cells transfected with GSK3β siRNA compared with cells transfected with non-tar-
geting siRNA (Fig. 1D). Although much of the low serum-induced CTPS1 phosphorylation was inhibited by GSK3β siRNA, the reduction was not as significant as that observed after indirubin treatment (Fig. 1C), suggesting that GSK3α may also contribute to the phosphorylation of CTPS1.

Identification of Ser-574 and Ser-575 as Phosphorylation Sites in Human CTPS1—To identify the amino acids phosphorylated in endogenous CTPS1, HEK 293 cells were labeled with [32P]orthophosphate and CTPS1 was isolated by immunoprecipitation. Tryptic peptides from endogenous CTPS1 were separated by two-dimensional TLC; the five phosphopeptide spots routinely observed were scraped and the peptides eluted and analyzed by nano-electrospray ionization tandem mass spectrometry. Spot 3 was identified as a carboxyl-terminal tryptic peptide (SGSS*S*PDSEITELK; the asterisk denotes phosphorylation) containing a phosphorylation on serine 575 (Fig. 2A). Spots 1 and 5 were identified as the same peptide isolated but the tandem mass spectrometry data were consistent with a doubly phosphorylated peptide (SGSS*S*PDSEITELK), Ser-574 and Ser-575 (Fig. 2A). The phosphopeptides were not identified from spots 2 and 4 possibly because the abundance of the phosphopeptide was too low, the ionization of these peptides was poor, or other peptides in the sample caused ion suppression. Analysis of the S574A mutant showed decreased intensity of spot 1 (Fig. 2B), substantiating the identification of this spot as a tryptic peptide containing phosphorylated Ser-574 and Ser-575. The S575A mutant showed decreased intensity of all the spots except for spot 3, which was barely detectable, suggesting that Ser-575 was a major phosphorylation site and/or that it was required to “prime” for additional phosphorylations (Fig. 2B). Mutation of Ser-574, Ser-575, or both Ser-574/Ser-575 to alanine (S574A, S575A, S574A/S575A) significantly decreased the

![Figure 2](image-url)
GSK3 Regulation of Human CTPS1

Involvement of Ser-574 and Ser-575 in GSK3β Phosphorylation of CTPS1—To determine whether GSK3β could phosphorylate Ser-574 and/or Ser-575 directly, CTPS1 or the indicated phosphorylation site mutants were immunoprecipitated and used as substrates in an in vitro kinase reaction with GSK3β (Fig. 2C). Both endogenous and transfected CTPS1 were found to be substrates for GSK3β, whereas by contrast the S574A, S575A, and the S574A/S575A mutants all showed greatly reduced phosphorylation by GSK3β. These data showed the ability of GSK3β to phosphorylate CTPS1 and indicated that Ser-574 and, to a greater extent, Ser-575 were involved in the direct phosphorylation of CTPS1 by GSK3β (Fig. 2C). Additionally, these phosphopeptides were separated by two-dimensional TLC to generate phosphopeptide maps and the pattern of spots compared with the pattern observed from CTPS1 phosphorylated in intact cells (compare phosphopeptide maps from Fig. 2A to phosphopeptides maps from Fig. 2C). These data strongly suggested that GSK3β was directly phosphorylating CTPS1 on sites similar to those observed in vivo.

Identification of Ser-571 as a Phosphorylation Site in Human CTPS1—GSK3 is a hierarchical kinase that requires prior phosphorylation of a serine/threonine (S/T) at a position n+4 from the GSK3 phosphorylation site (GSK3 consensus sequence, S/T-XXX(S/T-Phospho), where X is any amino acid) (22). The large decrease in phosphorylation observed with the mutation S575A, both in intact cells and in vitro with GSK3β, implicates Ser-575 as a critical residue involved in GSK3 phosphorylation of CTPS1. However, whether Ser-575 is serving as the actual GSK3 phosphorylation site or the priming site for GSK3 phosphorylation of Ser-571 could not be determined from the results in Fig. 2. To determine whether the negative charge of the glutamate at residue 579 (Glu-579) was serving as a phosphomimetic residue priming for GSK3 phosphorylation of Ser-575, this glutamate was mutated to alanine (E579A). Analysis of the E579A mutant in intact HEK 293 cells showed that mutation of this residue did not affect phosphorylation of CTPS1, demonstrating that Glu-579 does not prime for GSK3 phosphorylation of Ser-575 in intact cells (data not shown). Another possibility was that phosphorylation of Ser-575 could prime for GSK3 phosphorylation of Ser-571. Interestingly, the CTPS1 clone we received from the IMAGE consortium contained a mutation of Ser-571 to an isoleucine (S571I). To address whether Ser-571 was a phosphorylation site in CTPS1 in intact cells, the S571I mutation was expressed in HEK 293 cells labeled with [32P]orthophosphate. Following isolation by anti-FLAG immunoprecipitation, the CTPS1 S571I mutant had substantially less radioactivity associated with it compared with WT CTPS1-FLAG, demonstrating that Ser-571 was required for CTPS1 phosphorylation (Fig. 3A). The combined mutation of Ser-575 and Ser-574/Ser-575 to alanine with S571I decreased the radioactivity associated with CTPS1, suggesting that Ser-575 was another major site labeled under these conditions (Fig. 3A). Two-dimensional TLC revealed that when Ser-571 was mutated to an isoleucine, the majority of radioactive spots were diminished, with the exception of one prominent spot (Fig. 3A). This phosphopeptide spot did not disappear when Ser-574 was mutated to alanine in combination with S571I but was absent from the S571I/S575A double mutant, suggesting that the phosphopeptide that gives rise to this spot is dependent on Ser-575 being phosphorylated (Fig. 3A). Because serine 571 is mutated to a more hydrophobic residue (isoleucine), phosphorylated peptides containing this isoleucine would migrate farther in the second dimension of the two-dimensional phosphopeptide map but maintain approximately the same migration distance in the first dimension. This suggests that the prominent radioactive spot observed when Ser-571 was mutated to an isoleucine corresponds most likely to spot 2 or 3. Consistent with this, spot 3 was identified earlier as the same tryptic peptide containing unphosphorylated Ser-571 and singly phosphorylated Ser-575 (see Fig. 2A).

Involvement of Ser-571 in GSK3β Phosphorylation of CTPS1—To determine whether Ser-571 was a substrate for direct phosphorylation by GSK3, WT, S571I, S571I/S574A, S571I/S575A, and S571I/S574A/S575A were used as substrates in an in vitro kinase reaction with GSK3β. Incubation with GSK3β increased the phosphorylation of WT CTPS1 whereas the phosphorylation of S571I was significantly lower, demonstrating that Ser-571 was indeed a phosphorylation site for GSK3β (Fig. 3B). Analysis of the S571I/S574A phosphorylation site mutant did not show altered incorporation of phosphate compared with S571I alone, but analysis of the S571I/S575A mutant showed slightly elevated amounts of phosphate incorporated into CTPS1 compared with S571I, suggesting that without the ability to phosphorylate Ser-571 or Ser-575 in vitro, GSK3β may phosphorylate an alternative site, albeit to a much lesser extent. Analysis of the triple mutant S571I/S574A/S575A showed a slight decrease in the amount of phosphate incorporated into CTPS1 compared with S571I/S575A, suggesting that Ser-574 may serve as a minor secondary site for GSK3β phosphorylation in vitro (Fig. 3B). Separation of phosphorylated peptides from the in vitro GSK3β kinase reaction by two-dimensional TLC demonstrated that spots 1 and 5 were the most intense (see arrow in Fig. 3B). Although we previously found the doubly phosphorylated (Ser-574 and Ser-575) peptide in these two spots, this does not preclude the co-migration of other phosphorylated peptides, in particular phosphorylated peptides that are not as easily detected using mass spectrometry, such as a large multiply phosphorylated peptide similar to the one that would be generated if Ser-571 were phosphorylated. As expected, mutation of Ser-571 to isoleucine (S571I) abolished the appearance of this spot (see arrow in Fig. 3B), demonstrating that this spot contains phosphorylated Ser-571 and that Ser-571 is the major site in CTPS1 for GSK3β. Because the phosphorylation of CTPS1 was performed in vitro, only sites phosphorylated by GSK3β would incorporate radioactivity; other residues phosphorylated prior to the in vitro kinase reaction may still be phosphorylated but not labeled with radioactivity. Because GSK3β requires priming phosphorylation, this spot (Fig. 3B, arrow) contains a peptide with at least two phosphorylated residues, Ser-571 and Ser-575 (Fig. 3B). Further mutation of Ser-574, Ser-575, and both Ser-574/Ser-575 to alanine combined with S571I did not change the results.
observed with S571I alone, again demonstrating that Ser-571 was the major target of GSK3β in vitro.

The Effect of GSK3 Inhibition and Serum on CTPS1 Activity—Most GSK3 substrates are negatively regulated by GSK3 phosphorylation (23–33). To examine whether CTPS1 was regulated by GSK3 phosphorylation, endogenous CTPS1 was isolated by immunoprecipitation from HEK 293 cells maintained in 10% FBS, 0.1% FBS, or 0.1% FBS with the GSK3 inhibitor indirubin, and the amount of CTPS1 activity was assayed. Incubation of cells with low serum decreased CTPS1 activity compared with cells incubated with 10% FBS (Fig. 4A) and paralleled the effect of these treatments on CTPS1 phosphorylation. Incubation of cells in low serum in the presence of the GSK3 inhibitor indirubin preserved CTPS1 activity (Fig. 4A), consistent with GSK3 phosphorylation inhibiting the activity of CTPS1.

The Effect of Phosphatase Treatment on CTPS1 Activity—To further examine the effect of phosphorylation on CTPS1 activity, CTPS1 was isolated from low serum-incubated HEK 293 cells by immunoprecipitation, treated for 7.5, 15, or 30 min with calf intestinal phosphatase (CIP), and CTPS1 activity was measured (Fig. 4B). CTPS1 was significantly dephosphorylated after 15 min of incubation with CIP as seen by phosphorimaging of total isolated CTPS1 and in the two-dimensional phosphopeptide map (Fig. 4B). The decrease in spot intensity of multiple spots in the two-dimensional phosphopeptide maps following phosphatase treatment suggests that multiple residues, including Ser-571, Ser-574, and Ser-575, are being dephosphorylated. CTPS1 activity increased with the time of incubation with CIP, demonstrating that CTPS1 was more active following dephosphorylation of the enzyme (Fig. 4B).

DISCUSSION

Although phosphorylation regulation of yeast and human CTPS1 expressed in yeast has been investi-
GSK3 Regulation of Human CTPS1

Large scale analyses of the phosphoproteome identified numerous phosphorylation sites in the carboxyl terminus of CTPS1 (Ser-562, Thr-566, Tyr-567, Ser-568, Ser-571, Ser-573, Ser-574, Ser-575, Ser-578, and Thr-581) (34). Our studies confirmed the phosphorylation of Ser-571, Ser-574, and Ser-575. The fact that the other sites were not detected in our analysis suggests that the other sites may be minor phosphorylation sites or were not labeled under the conditions used in this study. Interestingly, tyrosine phosphorylation was found on CTPS1 using an anti-phosphotyrosine antibody but did not require Ser-571 to be phosphorylated (data not shown). Phosphoamino acid analysis of CTPS1 also paralleled the results of the large scale phosphoproteome analysis, demonstrating that serine was the most highly phosphorylated amino acid, followed by threonine, and that phosphorylation of tyrosine was below the limits of detection by radioactivity (data not shown). The observation that multiple phosphorylation sites are clustered in the carboxyl terminus of mammalian CTPS1 suggests

on their carboxyl terminus but does suggest that the regulation of mammalian CTPS1 by phosphorylation may be different in these organisms. The difference between the carboxyl terminus from yeast and human CTPS1 suggests that human CTPS1, and perhaps nucleotide metabolism, is regulated differently in humans than in yeast. Ser-571 is highly conserved in all the mammalian sequences examined and is even conserved in CTPS2, indicating the potential importance of this residue in the phosphorylation control of mammalian CTPS. In human and mouse CTPS, Ser-575 is found only in CTPS1 and not in CTPS2 whereas in both CTPS1 and CTPS2 from other mammals position 575 is conserved. The ability of position 575 to be phosphorylated in both CTPS1 and CTPS2 from other mammals may indicate a more redundant role for CTPS1 and CTPS2 in these organisms, whereas the ability for 575 to be phosphorylated only in human and mouse CTPS1, but not CTPS2, suggests different roles for CTPS1 and CTPS2 in humans and mice. Multiple lines of evidence point to the role of GSK3 to phosphorylate and regulate CTPS1. Inhibition of GSK3 with the inhibitor indirubin and siRNA revealed that GSK3 was involved in the low serum-induced phosphorylation of endogenous human CTPS1. Mutation of Ser-575 to alanine inhibited much of the radioactivity associated with CTPS1, and Ser-575 was investigated as a direct GSK3 phosphorylation site by mutating the potential priming site, Glu-579, to alanine (E579A). Because the E579A mutant failed to alter CTPS1 phosphorylation, Ser-575 was investigated as a priming site for phosphorylation of Ser-571. From these studies it was concluded that Ser-571 was the major site for GSK3 phosphorylation in vitro (Fig. 5). Interestingly, the CTPS1 clone we received from the IMAGE consortium, derived from a retinitis pigmentosa cell line, contained a naturally occurring mutation of Ser-571 to isoleucine (S571I), which suggests that phosphorylation of Ser-571 may play a role in diseases such as retinitis pigmentosa. The kinase responsible for phosphorylation of Ser-571 was not identified despite investigation of a variety of well known proline-directed kinases (e.g. ERK, c-Jun amino-terminal kinase (JNK), p38, and cyclin-dependent kinases).3

Large scale analyses of the phosphoproteome identified numerous phosphorylation sites in the carboxyl terminus of CTPS1 (Ser-562, Thr-566, Tyr-567, Ser-568, Ser-571, Ser-573, Ser-574, Ser-575, Ser-578, and Thr-581) (34). Our studies confirmed the phosphorylation of Ser-571, Ser-574, and Ser-575. The fact that the other sites were not detected in our analysis suggests that the other sites may be minor phosphorylation sites or were not labeled under the conditions used in this study. Interestingly, tyrosine phosphorylation was found on CTPS1 using an anti-phosphotyrosine antibody but did not require Ser-571 to be phosphorylated (data not shown). Phosphoamino acid analysis of CTPS1 also paralleled the results of the large scale phosphoproteome analysis, demonstrating that serine was the most highly phosphorylated amino acid, followed by threonine, and that phosphorylation of tyrosine was below the limits of detection by radioactivity (data not shown). The observation that multiple phosphorylation sites are clustered in the carboxyl terminus of mammalian CTPS1 suggests

3 M. J. Higgins and L. M. Graves, unpublished data.
that multiple kinases may be involved in phosphorylation regulation of CTPS1, including other hierarchical kinases such as casein kinase 1. Our data demonstrate a unique regulation of CTPS1 activity by GSK3 phosphorylation.

The crystal structures of bacterial CTPS have identified conserved amino acids that are proposed to be necessary for catalysis, dimerization, and tetramerization (35, 36). However, the carboxyl-terminal region containing Ser-571, Ser-574, and Ser-575 is not present in these three-dimensional structures, making it difficult to predict exactly how phosphorylation of the carboxyl terminus affects CTPS activity. Analysis of the hydrophobic/hydrophilic character of human CTPS1 using the Kyte-Doolittle hydrophobicity scale indicates that the carboxyl terminus is hydrophilic and is most likely solvent-exposed, possibly allowing for interaction with proteins that affect CTPS activity. Phosphorylation of yeast CTPS1 affects activity through altering the tetramerization of CTPS1 (37), providing a precedent for how phosphorylation of human CTPS1 may regulate CTPS activity. Phosphorylation of human CTPS1 on the carboxyl terminus could directly influence tetramerization (e.g. negative charges of phosphate could cause domains to shift, allowing for tetramerization) or phosphorylation of the carboxyl terminus could affect tetramerization indirectly, for instance by recruiting a protein (or displacing a protein) that alters tetramerization. Both 14-3-3 and WW domain-containing proteins such as Pin1 interact specifically with phosphorylated residues and can mediate changes in CTPS1 activity. Interestingly, a large scale analysis of HeLa cell proteins that interact with 14-3-3ζ identified CTPS as an interacting protein (38).

GSK3 was originally identified as the third kinase responsible for the phosphorylation of glycogen synthase, which decreased glycogen synthase activity (39, 40). Since this seminal discovery, multiple substrates and cellular roles of GSK3 have been elucidated. Many GSK3 substrates identified are involved in metabolism (e.g. glycogen synthase, acetyl-CoA carboxylase, insulin receptor substrate, pyruvate dehydrogenase), demonstrating a prominent role for GSK3 in regulating metabolism (reviewed in Ref. 41). The decrease in CTPS1 activity by GSK3 is consistent with GSK3 negatively regulating the function of other known GSK3 substrates (e.g. glycogen synthase, β-catenin, pyruvate dehydrogenase) and supporting the role of GSK3 phosphorylation as catalyzing inhibitory events. Interestingly, the carboxyl terminus of mammalian CTPSphosphocholine cytidylyltransferase (CCT, EC: 2.7.7.15), which is the rate-limiting enzyme in phosphatidylcholine biosynthesis and the next biosynthetic step in phospholipid metabolism, is phosphorylated on multiple sites like CTPS1 (42, 43). Moreover, CCT activity is decreased by phosphorylation (42, 43). Multiple phosphorylation sites in the carboxyl terminus of CCT are GSK3 consensus sites, suggesting that phosphatidylcholine, and possibly phospholipid synthesis in general, may be regulated by GSK3 phosphorylation of rate-limiting enzymes such as CCT and CTPS1. In yeast Carman and co-workers demonstrated the critical role of CTP and CTPS phosphorylation in regulating phospholipid synthesis (14, 44, 45). Hatch and McIarty (46) showed CTPS activity is critical for phospholipid synthesis in mammalian cells, supporting the notion that CTP synthesis and phospholipid synthesis could be coordinately regulated.

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