REGULATION OF HUMAN CYTIDINE TRIPHOSPHATE SYNTHETASE 1 BY PHOSPHORYLATION AND INTERACTING PROTEINS

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ABSTRACT

MATTHEW JAMES HIGGINS: Regulation of Human Cytidine Triphosphate Synthetase 1 by Phosphorylation and Interacting Proteins
(Under the direction of Dr. Lee M Graves)

CTP is essential for the synthesis of phospholipids, DNA/RNA and the sialylation of proteins. Of the two pathways known to synthesize CTP (*de novo* and salvage), the *de novo* synthesis pathway is thought to be the key regulator of CTP pool size in mammalian cells. CTPS is the rate-limiting enzyme in the *de novo* synthesis of CTP. CTPS catalyzes the amination of UTP to form CTP using glutamine as the amine donor and GTP as an allosteric activator. ATP is hydrolyzed in the process and CTP is a feedback inhibitor. In *S. cerevisiae* CTP synthetase (CTPS) is regulated by phosphorylation by PKA and PKC. The goals of this study were to investigate phosphorylation regulation of mammalian CTPS1 and elucidate CTPS1 interacting proteins. PKA or PKC were found not to be involved in the phosphorylation of CTPS1 in intact HEK 293 cells. However, endogenous human CTPS1 was phosphorylated under low-serum conditions in a GSK3-dependent manner. S571 was identified as the major GSK3 phosphorylation site and S574 and S575 were also identified as phosphorylation sites. GSK3 phosphorylation of human CTPS1 decreased enzyme activity and *in vitro* dephosphorylation increased CTPS1 activity indicating that phosphorylation suppresses CTPS1 activity. CK I was also found to be involved in the phosphorylation of CTPS1. CK I inhibition did not affect CTPS1 activity indicating that CK I may regulate another function of CTPS1. Investigation of CTPS1 interacting proteins revealed that the

peptidylprolyl isomerase, Pin1, can interact with CTPS1 in a S575 phosphorylation-dependent manner. However, reduction of Pin1 expression did not affect CTPS1 activity suggesting that the regulation of CTPS1 by Pin1 may be through another mechanism. Continued investigation of CTPS1 interacting proteins revealed, among other proteins, tubulin as a CTPS1 interacting proteins. Further investigation is needed to understand the significance of this interaction. CTPS1 expression was found to be highest in the brain suggesting CTPS1 may have a brain-specific function. This study is the first to characterize the phosphorylation regulation of mammalian CTPS1 expressed in mammalian cells and elucidate CTPS1 interacting proteins and CTPS1 expression in mammalian tissue.

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Completing a doctorate in philosophy is not possible without help and support from a great many great people. The first inclination for pursuing a PhD was seeded in me by Dr. Jakob Schmidt at SUNY Stony Brook, where I was a naive undergraduate doing some summer research before my senior year. After talking with a few graduate students, I sheepishly asked if Dr. Schmidt thought I should consider graduate school. Obviously, "yes" was the answer or else I wouldn't be writing this. To get enough research experience to convince admission committees to accept me as a graduate student I needed to prove I could do research. After graduating, I eventually found a good work environment in Dr. Zhengui Xia's laboratory at the University of Washington. With the training, guidance and the support of Dr. Xia and her husband Dr. Daniel Storm, I was prepared for being a successful graduate student. After interviewing at a variety of places I chose the University of North Carolina and settled on the laboratory of Dr. Lee M Graves in the department of pharmacology. Little did I know how challenging the CTPS1 project would be. But, with such a new project comes the mastery of a great deal of scientific skills that will be useful later in my career (or at least I hope). This project would not have been possible without the constant encouragement from Lee and his support when I needed it. I also think Lee had a vision of this project, which to a naïve graduate student, like myself, was difficult to comprehend. Also, this project would not have been possible without the help of my dissertation committee, giving excellent supporting guidance on experiments and general

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Since I'm not the best writer, scientist or leader I'll let those more proficient in those arts help explain, through some choice quotes, my perspective for pursuing science.

When scientific problems seem overwhelming:

> "Noli arrogantium iniurias pati" ('Don't let the bastards grind you down')
> Joseph "Vinegar Joe" Stilwell (1883-1946, 4-star General)

For unexpected results:

> "The most exciting phrase to hear in science, the one that heralds the most discoveries, is not"Eureka!" (I found it!) but "That's funny. . . " Isaac Asimov (1920-1992, Biochemist)

For ideas in opposition to the current dogma:

> "The farther the experiment is from theory, the closer it is to the Nobel Prize"
> Irene Joliet-Curie (1897-1956)
>
> "Everything we know is only some kind of approximation, because we know that we do not know all the laws yet. Therefore, things must be learned only to be unlearned again or, more likely, to be corrected." Richard Feynman (1918-1988, Physicist)

For the isolation of discovery:

> "What is there that confers the noblest delight? What is that which swells a man's breast with pride above that which any other experience can bring to him? Discovery! To know you are walking where none others have walked . . . "
>
> Mark Twain (1835-1910)
>
> "If we knew what it was we were doing, it would not be called research, would it?"
>
> Albert Einstein (1879-1955, Physicist)

Most important attribute or frame of mind for science:

> "When I examined myself and my methods of thought, I came to the conclusion that the gift of fantasy has meant more to me than my talent for absorbing positive knowledge." Albert Einstein (1879-1955)

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ADP	Adenosine 5'-diphosphate
ADPNP	5'-adenylyl β,γ-imidodiphosphate
$ADPCH_2P$	Adenosine-5'-[β,γ-methylene]triphosphate
AICAR	5-aminoimidazole-4-carboxamide riboside
AMP	Adenosine 5'-monophosphate
AMPK	AMP-activated protein kinase
AraC	Cytosine arabinoside
ATP	Adenosine 5'-triphosphate
ATPγS	adenosine 5'-(γ-thio)-triphosphate
CAD	Carbamyl phosphate synthetase, aspartate transcarbamylase and dihydroorotase
CPEC	Cyclopentenylcytosine
CTP	Cytidine triphosphate
dCTP	Deoxycytidine triphosphate
CTPS1	Cytidine triphosphate synthetase 1
DAU	3-deazauridine

DMSO	Dimethylsulphoxide
DON	6-diazo-5-oxonorleucine
Erk	Extracellular-signal regulated kinase
GS	Glycogen synthase
GSK3	Glycogen synthase kinase 3
HPLC	High pressure liquid chromatography
hCTPS1	Human CTPS1
IGF	Insulin-like growth factor
IRS	Insulin receptor substrate
ITC	Isothermal calorimetry
JNK	c-Jun N-terminal kinase
L. lactis	*Lactococcus lactis*
MAPK	Mitogen activated protein kinase
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
mTOR	Mammalian target of rapamycin

PCR	Polymerase chain reaction
PDK	3'-phosphoinositide-dependent kinase
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PRPP	5-phosphoribosyl-1-pyrophosphate
PtdIns-4,5-P_2	Phosphoinositol-4,5-bisphosphate
PtdIns-4-P	Phosphoinositol-4-phosphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
Thr	Threonine
Tyr	Tyrosine
VSMC	Vascular smooth muscle cell

CHAPTER

1. Introduction

1.1 CTPS Background

1.1.1 Discovery and History of CTPS

In 1956, Irving Lieberman reported that a partially purified bacteria extract catalyzed the amination of UTP to form CTP. The substrates required for the reaction were Mg^{2+}, UTP, NH_3, ATP and the products formed were CTP, ADP, and P_i (Lieberman, 1956). However evidence accumulated several years after this finding suggesting that glutamine is the source for the amination of UTP to form CTP. In particular, Edwin Sebrings group using ^{15}N labeled glutamine and ^{15}N labeled NH_4Cl demonstrated that the preferred source for the labeling of the cytosine ring in mammalian cells was glutamine and not NH_4Cl (Salzman et al., 1958). It was later suggested that the purification of the bacterial extract done by Lieberman et al. had inactivated the glutaminase activity of the enzyme, not allowing glutamine to serve as the amine donor and thus requiring NH_3 as a substrate. Around this same time Kammen and Hurlbert showed in lysates from rat hepatomas that low levels of guanosine nucleotides stimulated the conversion of uridine nucleotides to cytidine nucleotides two to three fold. In this same manuscript Kammen and Hurlbert also demonstrated that cytidine nucleotides had an inhibitory effect on the conversion of uridine nucleotides to cytidine nucleotides (Kammen and Hurlbert, 1958). Coupling these

observations with the original reaction proposed by Lieberman, produced a reaction where UTP, glutamine or NH_3, ATP, Mg^{2+} were required to form CTP and guanosine nucleotides stimulated the reaction and cytidine nucleotides, the reaction product(s), inhibited the reaction (Figure 1.1). The 300 fold purification of CTPS by Long and Pardee in 1967 allowed for the first kinetic studies of CTPS and revealed that GTP was the primary guanosine nucleotide involved in the stimulation of the reaction and CTP as the primary cytidine nucleotide involved in the feedback inhibition of the enzyme when substrates are at saturating concentrations (Long and Pardee, 1967).

With the advent of the molecular biology era it was possible to identify the genes encoding CTPS. The first mammalian CTPS gene, CTPS1, was identified by functional complementation of a cytidine auxotroph cell line with purified human metaphase chromosomes and was mapped to human chromosome 1 (Yamauchi et al., 1990). Since it was shown that the yeast genome encoded for two CTPS enzymes, van Kuilenburg et al. screened the NCBI database for a second CTPS isoenzyme, CTPS2, and identified an EST clone derived from colon tumor which was 74% identical to CTPS1 and mapped CTPS2 to the X chromosome (van Kuilenburg et al., 2000a; van Kuilenburg et al., 2000b).

1.1.2 Regulation of CTPS by Tetramerization

Initially, active CTPS was thought to exist as a dimer, because the molecular weight of the enzyme would double under certain conditions. Further investigation using CTPS purified from bacteria revealed that the doubling in molecular weight was due to the dimerization of dimers and not dimerization of monomers and that the isolated CTPS strongly associated as dimers and would form a tetramer when it was enzymatically active.

The minimum substrate requirement for purified *E. coli* CTPS to aggregate to the tetramer molecular weight (tetramer = 210,000 MW, monomer = 52,000 MW) was the presence of ATP, UTP and Mg^{2+} in the reaction mixture. UTP plus Mg^{2+} or ATP plus Mg^{2+} were not sufficient to cause aggregation to the tetramer molecular weight and NaCl concentrations up to 0.5 M did not alter the tetramerization of purified *E. coli* CTPS. Only under denaturing conditions (e.g. 6 M guanidine, 8 M urea or 60% formic acid) was the monomer observed, under all other conditions CTPS existed as a dimer or tetramer (Long et al., 1970). Unlike the tetramerization of *E. coli* CTPS, the tetramerization of *Lactococcus lactis* CTPS was found to coordinately decrease with higher concentrations of salt (NH_4Cl or KCl) suggesting that the influence of salt on oligomerization of CTPS my be species specific (Willemoes and Larsen, 2003).

The first investigation of the size and polymerization of mammalian CTPS was done by McPartland and Weinfeld in 1976 who found that CTPS purified from bovine liver existed as a 130,000 molecular weight enzyme in the absence of nucleotides and a 260,000 molecular weight protein in the presence of UTP, ATP and GTP. UTP and ATP were absolutely required for the formation of the 260,000 MW species (Long et al., 1970). In 1988, CTPS was purified 31,000 fold from rat liver cytosol and by HPLC and SDS-PAGE analysis mammalian CTPS was found to exist as a monomer (66,000 MW), dimer (120,000 MW) and a tetramer (240,000 MW). Incubation of the dimer with UTP, ATP and $MgCl_2$ were found to be the optimum conditions by which the dimer formed the tetramer recapitulating the conditions by which the bacterial CTPS also formed a tetramer (Thomas et al., 1988).

Investigation of CTPS tetramer formation from *Saccharomyces cerevisiae* (*S. cerevisiae*) revealed a requirement for ATP, UTP and $MgCl_2$ consistent with the conditions for CTPS tetramer formation in bacteria and mammals. However, elimination of ATP did not entirely inhibit tetramer formation suggesting that ATP facilitates but is not required for tetramer formation in *S. cerevisiae*. Furthermore, using the non-hydrolyzable ATP analog, AMP-PNP, the tetramer formation in *S. cerevisiae* was inhibited demonstrating that ATP needs to be hydrolyzed for ATP to fully enhance tetramerization. Elimination of UTP completely inhibited tetramerization demonstrating the requirement for UTP in CTPS tetramerization. Tetramerization of CTPS in the presence of saturating concentrations of ATP and UTP was not affected by varying protein concentration from 0.1 to 1 mg/ml (Pappas et al., 1998).

1.1.3 Feedback Regulation of CTPS

Feedback inhibition is a common mode for regulating enzymes. CTPS generates three products; glutamate, ADP and CTP. Glutamate, the product formed from the deamination of glutamine, was not observed to inhibit calf liver purified CTPS when the concentrations of UTP, ATP or glutamine were varied.

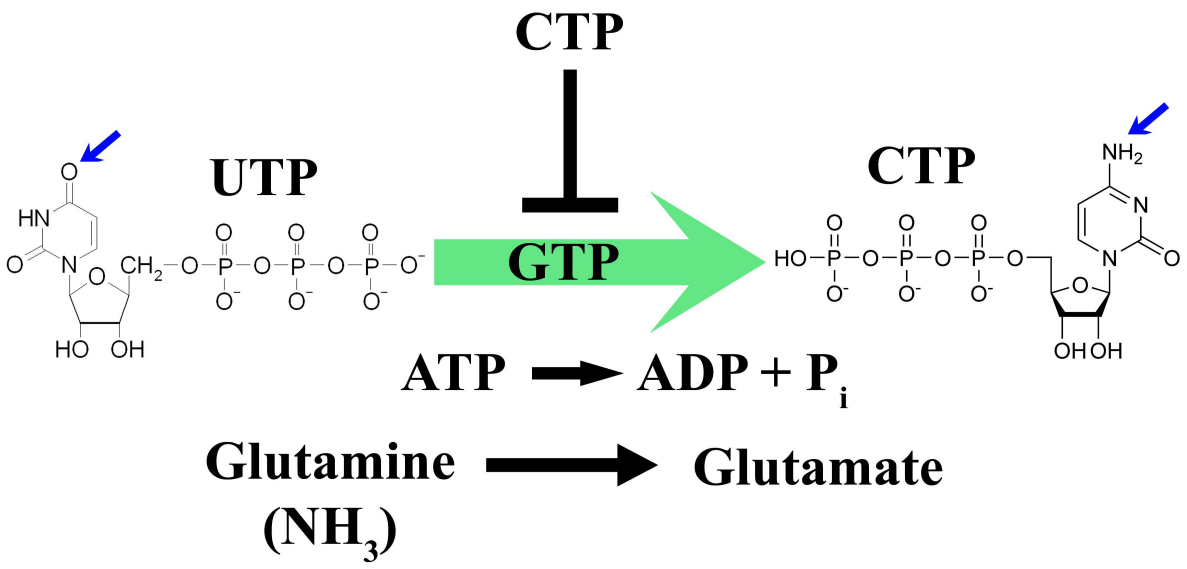


Figure 1.1 CTPS Reaction Mechanism

Blue arrow designates site on UTP pyrimidine ring where amine is exchanged. Glutamine is the amine donor that gets attached to 4 position on UTP to form CTP. CTP is a feedback inhibitor and GTP allosterically activates CTPS. ADP and glutamate are byproducts of the CTPS reaction.

ADP, the product of ATP hydrolysis, showed mixed (competitive and non-competitive) inhibition with respect to ATP and UTP, but uncompetitive inhibition with respect to glutamine which led to the suggestion that ADP may need to be released from the enzyme before UTP or glutamine can bind (McPartland and Weinfeld, 1979).

As observed with other enzymes, the major product of the CTPS reaction, CTP, can feedback inhibit CTPS. In CTPS purified from *E. coli*, CTP was found to be a competitive inhibitor with respect to UTP when ATP concentrations were saturating and a non-competitive inhibitor with respect to ATP when concentrations of UTP were saturating. CTP inhibition was observed when either NH_3 or glutamine served as amine donors. When glutamine was used as the amine donor and the concentrations of UTP or ATP are non-saturating, CTP (0.3 mM) also acted as an inhibitor and did not markedly affect the cooperative binding of ATP or UTP. When NH_3 was used as the amine donor and the concentrations of UTP or ATP were non-saturating, CTP (0.3 mM) was activating and reduced the cooperative binding of both UTP and ATP. The addition of CTP in the NH_3 reaction changed the sigmoidal kinetics of UTP and ATP to Michaelis kinetics. At higher concentrations of CTP in the NH_3 reaction CTP was found to be less activating and eventually inhibitory (Long and Pardee, 1967). The ability of CTP to activate the CTPS reaction under conditions where substrates are not saturating is thought to insure basal synthesis of CTP.

Investigation of CTP inhibition of CTPS purified from calf liver using glutamine as the amine donor revealed that CTP showed mixed (competitive and non-competitive) inhibition with respect to glutamine and ATP. CTP inhibition with respect to UTP shifted

the kinetics from Michaelis kinetics to sigmoidal kinetics suggesting increased cooperativity in the presence of CTP. Similar to what was observed with the bacterial CTPS, lower concentrations of CTP (0-0.4 mM) were activating and higher concentrations were inhibitory. In contrast to CTP, 3-deaza UTP, a competitive inhibitor with respect to UTP, showed inhibition at all concentrations. CTP did not alter the oligomerization of purified calf liver CTPS further demonstrating that CTP feedback inhibition of CTPS does not involve dissociation of the CTPS tetramer (McPartland and Weinfeld, 1979).

Analysis of CTP feedback inhibition in *S. cerevisiae* demonstrated that, like mammalian CTPS, yeast CTPS was inhibited by CTP with respect to UTP and the presence of CTP increased the cooperativity with respect to UTP. CTP decreased the apparent V_{max} and increased the apparent K_m for UTP (Yang et al., 1994). CTP also did not prevent tetramerization of CTPS demonstrating that the mechanism by which CTP inhibits CTPS does not involve inhibiting tetramerization (Pappas et al., 1998; Yang et al., 1994).

1.1.4 GTP Regulation of CTPS

One of the first observations that guanosine nucleotides, including GTP, could positively regulate CTPS was published in 1960 by Hurlbert and Kammen who found that CTPS, purified from Novikoff transplantable hepatoma cells, could be stimulated by guanosine nucleotides and that GTP could stimulate the amination activity of CTPS up to 500% (Hurlbert and Kammen, 1960). Using CTPS purified from *E. coli*, GTP at non-saturating concentrations was not found to cause cooperative binding with respect to UTP suggesting that GTP, unlike ATP at non-saturating concentrations, did not influence UTP binding and the effect of GTP on CTPS activity was most likely not by altering UTP binding

(Long and Pardee, 1967). Further investigation by another group also using purified bacteria CTPS found that GTP enhanced the negative cooperativity with respect to glutamine when the concentration of GTP was progressively decreased below the saturating concentration of GTP implicating GTP in regulating the glutaminase half of the CTPS reaction and not the half involving ATP and UTP (Levitzki and Koshland, 1969). UTP or ATP binding cooperativity was not observed with CTPS purified from bovine liver when the concentration of GTP was varied, suggesting that in mammalian CTPS, as in bacterial CTPS, GTP does not increase CTPS activity by influencing ATP or UTP binding (Savage and Weinfeld, 1970). With evidence accumulating that GTP was not affecting the ATP and UTP half of the CTPS reaction, future investigations focused on the glutamine half of the reaction. DON, the glutamine transition state analog, reacts with one of the two subunits of the CTPS dimer and the presence of saturating concentrations of GTP increased the reaction rate up to eight fold. When saturating concentrations of GTP were added to the CTPS tetramer, formed by addition of UTP and ATP, the rate of reaction with DON was still increased eight fold, as was observed with the GTP and the CTPS dimer, but the negative cooperativity of the DON reaction with the tetramer was abolished (Levitzki et al., 1971).

A thorough analysis of the allosteric effect of GTP on CTPS activity was done using non-hydrolyzable ATP analogs (ADPNP, $ADPCH_2P$) which inhibits the ATP and UTP reaction, but allow the glutaminase reaction to proceed. Using ADPNP, GTP activated the glutaminase activity (increase in k_{cat}, increase in the turnover of molecules) to the same extent as with ATP. The increase in turnover number (k_{cat}) of the glutaminase reaction using the tetramer was appreciably higher than that of the dimer and approximated the amount of CTP formed suggesting that the NH_3 is consumed as soon as it is formed. The glutaminase

activity of CTPS is much less than the activity of another glutaminase that had been investigated (CTPS k_{cat} = 290 sec^{-1}, glutaminase k_{cat} = 38,000 sec^{-1}) further suggesting that the glutaminase activity in CTPS is there for providing NH_3 for the formation of CTP and not a general cellular glutaminase. Binding of GTP at 25°C to CTPS in the presence of UTP and $ADPCH_2P$ or in the presence of UTP and ATP with DON labeled CTPS showed distinct negative cooperativity indicating that under conditions where the formation of the tetramer is favored, GTP binding negatively influences further binding of another GTP molecule. However, GTP binding to DON-labeled CTPS in the presence of UTP and ATP at 4°C showed positive cooperativity indicating that temperature can influence GTP binding. GTP binding to the dimer at 25°C was found to be independent, not cooperative, and GTP bound to two sites in the dimer. The allosteric effect of GTP on CTPS activity when NH_3 was used as the substrate instead of glutamine showed that GTP inhibited the NH_3 activity of CTPS only when CTPS was labeled with DON otherwise GTP had no effect on CTPS when NH_3 was used as a substrate (Levitzki and Koshland, 1972).

Characterization of GTP regulation of *S. cerevisiae* CTPS revealed that GTP was not an absolute requirement for CTPS activity, but GTP did stimulate activity four fold. Negative cooperativity was observed with respect to both glutamine and GTP, especially at lower concentrations consistent with what has been observed before (Levitzki and Koshland, 1969) (Yang et al., 1994). Investigation of human CTPS partially purified from a leukemia cell line, HL60, demonstrated that GTP showed slight positive cooperativity in the presence of saturating concentrations of UTP and ATP (Van Kuilenburg et al., 1998).

The most recent biochemical investigation of how GTP affects CTPS activity was done using CTPS purified from *L. lactis*. To uncouple CTP synthesis from the glutaminase activity the non-hydrolyzable ATP analog, ATPγS, was used. In the presence of ATPγS, GTP can cause up to a six fold increase in k_{cat} as measured by isothermal calorimetry (ITC), but the K_m for glutamine remained the same. This indicates that GTP increases the turnover rate of the glutaminase reaction, but does not alter the affinity of CTPS for glutamine. At GTP concentrations higher than 1 mM and UTP plus ATPγS concentrations at 0.1 mM, GTP was inhibitory and this inhibition disappeared when UTP plus ATPγS concentrations were increased to 1 mM suggesting UTP and possibly ATP binding influence the effect GTP has on the glutaminase activity of CTPS. Coupling CTP synthesis with glutaminase activity by substituting 1 mM ATP for 1 mM ATPγS, resulted in GTP (1 mM) stimulating a 1.7 fold increase in the k_{cat} for hydrolysis of glutamine, but a forty-one fold increase in k_{cat} for CTP synthesis and a slight decrease in the K_m for glutamine when compared to the absence of GTP. Further investigation using 0.1 mM UTP and 0.1 mM ATP demonstrated only a seven fold increase in k_{cat} for CTP synthesis and a K_a value for GTP three orders of magnitude lower than what was observed when the reactions were uncoupled (i.e. in the presence of ATPγS instead of ATP). Coupling of the CTP synthesis reaction with the glutaminase reaction greatly enhances the ability of GTP to positively regulate CTPS. Indeed, the authors found that in the absence of GTP, the glutamine hydrolysis rate was significantly higher than the rate of CTP synthesis and the presence of GTP seemed to couple the glutaminase reaction with CTP synthesis. Based upon the accumulation of data the authors suggest that the true activator of the CTPS reaction is the fairly stable 4-phosphorylated UTP intermediate. Also, as observed with *E. coli* CTPS, these authors observed that DON-labeled CTPS was inhibited

by GTP when NH_4Cl was used as a substrate. However, inhibition of *L. lactis* CTPS by GTP showed negative cooperativity whereas the *E. coli* enzyme did not. These authors support a model where GTP acts as a "lid" that functions to coordinate glutamine hydrolysis with the formation of 4-phosphoryl UTP possibly by playing a role in the formation of the tunnel whereby the liberated NH_3 is passed to the active site containing the 4-phosphoryl-UTP intermediate (Willemoes and Sigurskjold, 2002).

1.1.5 CTPS Reaction Mechanism

The elucidation that CTPS catalyzed the formation of CTP by aminating UTP using glutamine as the preferred amine donor and hydrolyzing ATP in the process suggested CTPS catalyzed two separate, but coordinately regulated reactions. The first reaction was the liberation of NH_3 from glutamine catalyzed by the glutaminase region of CTPS and the second reaction was the hydrolysis of ATP which involved UTP.

1.1.5.1 Role of UTP and ATP

Early biochemical studies demonstrated that UTP concentrations could influence ATP binding and ATP concentrations could influence UTP binding suggesting these two substrates had interacting binding sites (Long and Pardee, 1967). To investigate whether a phosphorylated UTP intermediate was formed, UTP was labeled with [^{18}O] at the 4' position of the pyrimidine ring of UTP, the position of the pyrimidine ring on UTP that gets aminated to form CTP, and used in a CTPS reaction. P_i containing [^{18}O] was found as a product of the reaction demonstrating that a 4'-phosphoryl UTP intermediate is formed (Levitzki and Koshland, 1971). Since the exchange of ADP for ATP was very low (only ~1%) in the absence of glutamine or ammonia and the exchange did not depend on UTP, and the nascent

ammonia liberated from glutamine was thought to immediately react with UTP, the model championed by many researchers at the time was that ammonia reacted with UTP to form a NH_2-UTP adduct that then reacted with ATP to form a H_2PO_4-NH_2-UTP tetrahedral intermediate. Since the phosphate molecule on the 4' position is an excellent leaving group and the tetrahedral intermediate is not very stable, CTP is formed in an almost irreversible fashion (Koshland, 1974).

Using the positional isotope exchange (PIX) technique developed by Midelfort and Rose, researchers revisited the investigation of intermediates in the CTPS reaction. Using ATP labeled with [^{18}O] at the β-γ bridge position it was shown in the absence of ammonia but in the presence of UTP and Mg^{2+} that UTP can be phosphorylated at the 4 position on the pyrimidine ring of UTP (von der Saal et al., 1985a; von der Saal et al., 1985b). Furthermore, the data suggested that ADP release occurred only after ammonia binding revealing that ADP binds tightly to CTPS and could explain why the ATP-ADP exchange in the absence of ammonia was not observed in the initial studies by Koshlands group. Using CTPS partially purified from Ehrlich ascites tumor cells it was shown that in the absence of substrates ADP is not formed, further demonstrating the requirement for all substrates being present before ADP can be exchanged (Kizaki et al., 1985). Using rapid quench kinetics it was demonstrated that GTP increases the rate of the steps of the reaction leading up to intermediate formation and glutamine was also found to shift the internal equilibrium towards the formation of the intermediate complex. Furthermore, in the absence of GTP data was obtained, using isotope partitioning, suggesting that UTP dissociates from the enzyme complex faster than it is used to form products. In the presence of GTP more of the enzyme-UTP complex was formed suggesting GTP increases the rate of product formation relative to

substrate dissociation. Combining the data from the rapid quench kinetic and isotope partitioning experiments demonstrates that ATP phosphorylates UTP to generate a 4-phosphoryl UTP intermediate and this intermediate plays a key role in determining the rate of the CTPS reaction (Lewis and Villafranca, 1989).

1.1.5.2 Role of the Amine Donor, Glutamine

Ammonia can serve as the amine donor directly for the formation of CTP, but since the concentrations of exogenous ammonia needed to achieve appreciable enzyme activity are high, this is thought, for most organisms, not to be biologically relevant. Evidence soon after the discovery of the CTPS reaction accumulated that glutamine seemed like the preferred amine donor. In particular early work done by Salzman using [^{15}N] labeled glutamine and Eidinoff who found that the glutamine inhibitor, DON, inhibited the cytosine incorporation in DNA and RNA provided the first evidence suggesting that glutamine serves as the amine donor for the CTPS reaction (Eidinoff et al., 1958; Salzman et al., 1958). Many studies since this seminal discovery have confirmed that glutamine is the preferred amine donor. However, one study using CTPS partially purified from Chinese hamster fibroblasts has found synergism between amine donors and also found that arginine can serve as an amine donor (McLaren and Chu, 1983). The same synergy between amine donors and whether arginine can serve as an amine donor has not been investigated in other organisms suggesting glutamine may not be the only amine donor or that the hamster CTPS is biochemically different from other CTPSs that have thus far been investigated.

The reaction that catalyzes the release of ammonia from glutamine occurs in the glutaminase region of CTPS. Initial investigation of the number of glutamine binding sites in

CTPS using [^{14}C] labeled DON revealed one molecule of DON bound per CTPS dimer (Long et al., 1970). A more thorough investigation found that CTPS reacts through a "Half-of-the-Sites" reaction mechanism meaning that only half of the potential substrate binding sites are occupied when substrates or inhibitors are at saturating concentrations (e.g. total substrate binding sites = n , during substrate/inhibitor saturation only n/2 are occupied) (Levitzki et al., 1971). GTP can affect DON binding, but at saturating concentrations of DON the "Half-of-the-Sites" reaction mechanism is still observed.

The CTPS glutaminase domain belongs to the family of type I amidotransferases also known as the triad type because they contain a cysteine-histidine-glutamate triad of amino acid that catalyze glutamine hydrolysis. Other members of the triad type of amidotransferases include anthranilate synthase, formylglycinamidine synthase, GMP synthase, imidazole glycerol phosphate synthase and carbamoyl phosphate synthase (CPS) (Mei and Zalkin, 1989; Zalkin, 1985; Zalkin, 1993). Mutation of the catalytic cysteine (C379) in *E. coli* CTPS, identified by sequence alignment with other amidotransferases, to a serine or alanine reduced the apparent K_m and k_{cat} of glutamine below detectable levels whereas the ability of the enzyme to used exogenous ammonia was unchanged (Bearne et al., 2001). The liberated ammonia molecule from glutamine hydrolysis travels down what is known as an ammonia tunnel where it reacts with the 4-phosphoryl UTP to form CTP. Limited proteolysis of the *E. coli* CTPS has identified that the amidotransferase domain resides in the c-terminus of the enzyme (Simard et al., 2003).

1.1.6 Structural Studies on CTPS

Thus far crystal structures from *E. coli* CTPS, *Thermus thermophilus* HB8 CTPS and the synthetase domain (amidoligase domain) of human CTPS1 have been solved. These structures have confirmed and clarified much of the biochemical evidence that has accumulated over the preceding half a century.

1.1.6.1 CTPS Oligomerization

The first published crystal structure of CTPS was that of *E. coli* CTPS without any ligands bound, apo CTPS. The high concentration of CTPS used for the crystallization induced tetramerization of CTPS allowing for the analysis of what residues are involved in both dimer and tetramer contacts (Figure 1.2). The dimer contacts of each monomer reside primarily in the N-terminal domain which contains the amidoligase domain where UTP is phosphorylated by ATP. 60% of the residues involved in the dimer-dimer contact are nonpolar and half of the buried nonpolar residues reside in a helix-loop-helix interdimer contact loop (residues 93-130, *E. coli* numbering). The strong association through buried nonpolar dimer-dimer contacts is one explanation of how CTPS can be found primarily as a soluble dimer when inactive (Endrizzi et al., 2004).

In the apo structure of CTPS from *E. coli*, large "trenches" between the association of two dimers (tetramer) were noted by the authors. The contact sites of two dimers and the formation of the "trenches" corresponded to the amidoligase domain of CTPS. Comparing the structure of the amidoligase domain of CTPS to that of the most structurally similar protein (dethiobiotin synthetase) and the highest conservation of residues in this area contains

three anion-binding sites (i.e. phosphate binding sites) allowed for the modeling in of the ATP and UTP binding sites, which modeled to the "large" trenches between the two dimers.

The walls of the ATP and UTP binding sites were contributed by residues from three of the subunits in the tetramer, providing evidence of how ATP and UTP can induce tetramerization of CTPS (Figure 1.3). The authors use the appropriate analogy that ATP and UTP are like "mortar" holding the tetramer of CTPS together (Endrizzi et al., 2004). One of the crystal structures of *T. thermophilus* had three SO_4^{2-} bound, which mimics the binding of phosphates, and allows the modeling of ATP and UTP into the CTPS structure. Based upon where the ATP and UTP were bound, required contacts from three of the four subunits further supporting the idea that ATP and UTP act as a "mortar" for tetramer formation. The residues identified for coordinating the phosphates were well conserved from a variety of species providing further evidence that ATP and UTP do bind to the tetramer of CTPS in this fashion (Goto et al., 2004). Also, the need for ATP and UTP binding for tetramer formation could explain the cooperative binding observed for ATP and UTP in some of the earlier biochemical studies.

1.1.6.2 CTP Binding Site

The CTP binding site in the *E. coli* apo CTPS was determined by identifying residues in the CTPS structure that corresponded to residues in *Chlamydia trachomatis* and Chinese hamster CTPS that when mutated were insensitive to CTPS inhibitors, had decreased CTP inhibition and increased UTP amination activity (Whelan et al., 1993; Wylie et al., 1996). Based upon the location of these residues, CTP bound to the CTPS tetramer in such a way that the residues that interact with the phosphates of CTP also are the same residues that

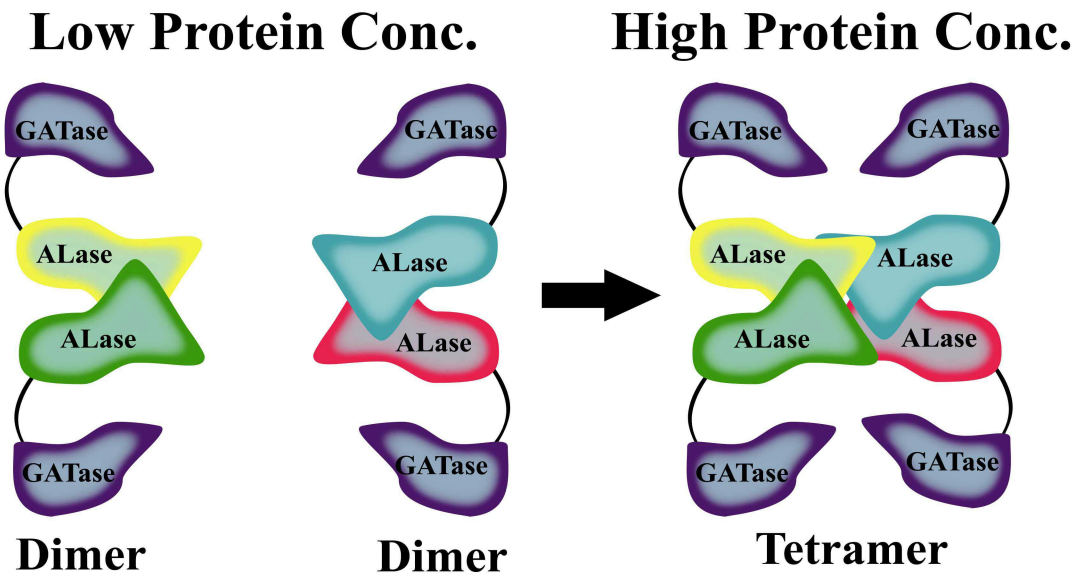


Figure 1.2 Model of Concentration Dependent Tetramerization of CTPS

Two dimers form a tetramer under high concentration of CTPS protein. Amidoligase domain (ALase, N-terminus), glutamine amidotransferase (GATase, C-terminus) and flexible linker connecting both domains are shown. Note how less compact the tetramer is compared to the tetramer in Figure 1.3.

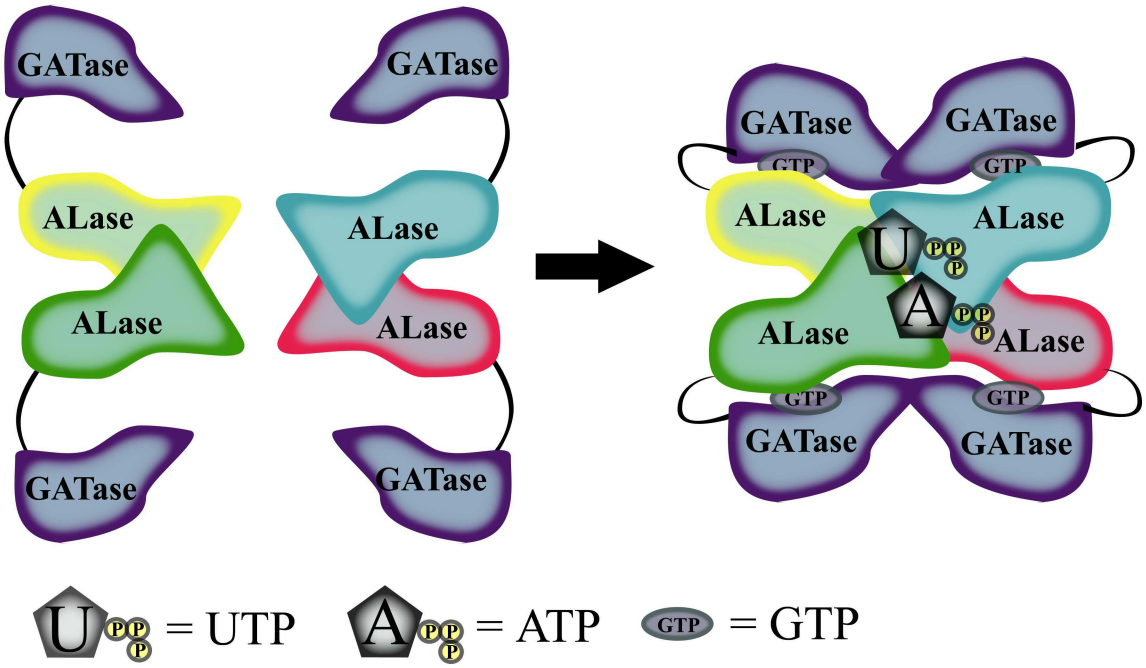


Figure 1.3 Model of Ligand-induced CTPS Tetramer Formation

UTP and ATP bind to the tetramer interface solidifying the tetramer. GTP is thought to bring the GAtase (glutamine amidotransferase domain) in closer contact with the ALase (amidoligase domain). Note how more compact the tetramer is compared to the concentration induced tetramer (Figure 1.2).

interact with the phosphates of UTP, demonstrating how CTP could compete with UTP binding and thus inhibit CTP synthesis (Endrizzi et al., 2004).

In support of this model a crystal structure of *E. coli* CTPS in complex with CTP found that CTP bound as predicted previously. Furthermore, the CTP-CTPS co-crystal structure demonstrated that multiple subunits of CTPS make contact with CTP, similar to how UTP is thought to bind. The contact of CTP with multiple subunits may explain how CTP, especially at lower concentrations of CTP, can enhance activity. The same analogy describing ATP and UTP as "mortar" holding together the tetramer was used by the same authors for how CTP can be promoting CTP synthesis under some conditions (e.g. low concentration of CTP) by promoting tetramerization (Endrizzi et al., 2005).

1.1.6.3 GTP Binding Site

A possible GTP binding site in apo *E. coli* CTPS was identified by comparing the glutamine amidotransferase domain of CTPS with that of other GTP binding proteins (EF-Tu and EF-G). The proposed GTP binding site was found near a cleft adjacent to the supposed entrance to the glutaminase site. The proposed GTP binding site contains a 3Å opening which would allow solvent access to the glutaminase reaction site. The binding of GTP is proposed by the authors to occlude this 3Å opening and block access of solvent molecules such as exogenous ammonia to the glutamine amidotransferase region of CTPS. This GTP binding site in CTPS is similar to the "lid" structure found in other glutamine amidotransferases (e.g. CPS) sealing the glutaminase region of the enzyme from exposure to

the solvent. It is also proposed that GTP binding may bring the catalytic residues required for glutamine hydrolysis closer to the glutamine for optimal catalysis (Endrizzi et al., 2004).

Using data from mutational analysis and sequence conservation (Iyengar and Bearne, 2003; Simard et al., 2003; Willemoes, 2003) a GTP binding site was suggested in the crystal structure from *T. thermophilus* (Goto et al., 2004). The GTP binding site corresponds to the same "lid" region of the glutamine amidotransferase domain described in the *E. coli* structure, but also includes residues from the amidoligase domain as well. The opportunities for the glutamine amidotransferase domain and amidoligase domain to come in close enough proximity to bind GTP is increased when both UTP and ATP are present suggesting that GTP binding may act to stabilize the conformation where the glutamine amidotransferase domain and the amidoligase domain are in close contact (Figure 1.3). Although the authors fail to use the mortar analogy that was used to describe how ATP and UTP stabilize tetramer formation, the same analogy could be used to describe how GTP holds the amidotransferase domain and the amidoligase domain together thus increasing CTPS activity. The model of GTP binding proposed, based on crystal structures from *T. thermophilus* and *E. coli*, is supported by mutation of these residues predicted by the crystal structures to be involved in GTP binding (Willemoes et al., 2005).

1.1.6.4 Glutamine Amidotransferase Domain

The glutamine amidotransferase (GATase) domain is found in other glutamine hydrolyzing enzymes including carbamoyl phosphate synthetase (CPS), which is involved in uridine biosynthesis. The GATase domain of CTPS contains many conserved residues found in other GATases domains of the same family. The triad family of GATase domains, of

which the CTPS GATase domain is a part of, is defined by the catalytic triad of cysteine-histidine-glutamate. Superimposing the GATase domain of CTPS on CPS reveals that Cys379, His515 and Glu517 (*E. coli* numbering) are the triad catalytic residues in *E. coli* CTPS. In all triad family of glutaminases there exists an "oxyanion hole" for the stabilization of the tetrahedral transition state of the glutaminase reaction (i.e. stabilizes the sidechain carboxylate on glutamine). In the crystal structure of apo CTPS from *E. coli* this "oxyanion hole" was proposed to be formed by the amides of the main peptide chain of residues Gly352 and Leu380 (Endrizzi et al., 2004). The formation of the "oxyanion hole" by these residues was confirmed in the *T. thermophilus* structure of glutamine-bound CTPS. In addition to confirming the catalytic triad residues suggested in the *E. coli* apo crystal structure, the glutamine-bound *T. thermophilus* crystal structure revealed that Tyr64, part of the amidoligase domain, and His522 and Arg470 (*T. thermophilus* numbering) coordinate a water molecule, which is proposed to be in favorable orientation for catalytic action (e.g. hydrolysis of glutamine) (Goto et al., 2004). Interestingly, in the *T. thermophilus* glutamine-bound crystal structure of CTPS a second His-Glu pair (His471 and Glu474, *T. thermophilus* numbering) is found in close proximity to the catalytic cysteine and is suggested by the authors to also be involved in catalysis. The distances between the histidine residues and the catalytic cysteine in CTPS are larger than the distances found in other triad family of GATases (including CPS) (Thoden et al., 1999) except for anthranilate synthase (Knochel et al., 1999) suggesting that one possible mechanism by which GTP could increase glutaminase activity would be by bringing the catalytic residues in closer proximity to the substrate (Goto et al., 2004).

Also observed in GATases of other family members is an ammonia tunnel where the liberated ammonia exits the catalytic site and in the case of CTPS is passed to the amidoligase domain to form CTP. In the apo structure of CTPS from *E. coli* a relatively large solvent-filled "vestibule" was identified that connects the GATase domain to the amidoligase domain via the ammonia tunnel. A conserved insert in CTPS and not found in the GATase domain of CPS, amino acids 297-301 (*E. coli* numbering), contributes to the wall of the vestibule and creates this opening by diverting sidechains that would normally occlude this opening in CPS. The vestibule contains a number of hydrogen bonding groups that could coordinate the liberated ammonia into the ammonia tunnel, which ultimately leads to the amidoligase domain where the 4-phosphoryl UTP can be found. The vestibule is also solvent accessible through the 3Å opening, where GTP is proposed to bind suggesting that when GTP and glutamine are both bound ammonia cannot access the vestibule and this limited access is thought to be the mechanism by which GTP inhibits CTPS activity when exogenous ammonia is used as a substrate (Endrizzi et al., 2004). Connected to the vestibule is a passage way that exits to the amidoligase active site in close proximity to where the 4-phosphoryl UTP intermediate is thought to reside. There exists a restriction point between the vestibule and the proposed exit composed of Pro54 and Val60 (*E. coli* numbering), which the authors suggest is a gate which allows access of the ammonia from the vestibule/ammonia channel to the amidoligase domain. Two conformations of His57 (*E. coli* numbering) located in the amidoligase domain were noted by the investigators. One conformation of His57 has its side chain next to where UTP is thought to bind and the other conformation occludes the exit from the vestibule/ammonia tunnel suggesting that UTP binding may alter the conformation of His57 such that ammonia is allowed to enter the

amidoligase domain and react with the 4-phosphoryl UTP intermediate (Endrizzi et al., 2004).

The structure of CTPS from *T. thermophilus* that has three sulfates and glutamine bound contains a Tyr64 (*T. thermophilus* numbering) from the amidoligase domain that interacts with the amide group of glutamine and is suggested by the authors to act as a door whereby the nascent ammonia released from the active site can enter the ammonia tunnel. The gating of this door is thought to be ATP and UTP dependent such that when ATP and UTP are bound, ammonia is allowed into the ammonia tunnel (Goto et al., 2004). This is the same residue (Tyr64) projected to be involved in orienting the water molecule for glutamine hydrolysis, suggesting UTP and ATP binding may alter the orientation of Tyr64 to more favor glutamine hydrolysis. Having UTP and ATP controlling a critical residue in the glutaminase region of CTPS allows the coupling the glutaminase reaction with the binding of substrates (ATP and UTP) needed for the next catalytic step in CTPS.

1.1.6.5 Amidoligase Domain

The amidoligase domain, which is located on the N-terminus of CTPS, binds UTP and ATP and catalyzes the phosphorylation of UTP to the 4-phosphoryl UTP intermediate followed by nucleophilic attack by ammonia on the four position of UTP to generate CTP. Additionally, many of the contact sites for tetramer formation reside in the amidoligase domain (Figure 1.3). The amidoligase domain most closely resembles that of dethiobiotin synthetase (DTBS) and the folds of the kinase domain within the amidoligase domain most closely resemble members of the ATP/GTPase superfamily such as APS kinase (Endrizzi et al., 2004). The active site for the amidoligase domain resides between the binding site for

the two homodimers and residues from multiple subunits contribute to ATP and UTP binding (Figure 1.3). Unfortunately, thus far crystal structures with sulfates bound have been solved and the binding sites for UTP and ATP are modeled into the CTPS structure based on the location of ATP and UTP other structurally similar proteins as well as the location of the sulfates, which does not allow for a rigorous interrogation of how the amidoligase domain may function.

1.1.6.6 Reaction Mechanism and Conformational Change

Evidence from the crystal structures from apo, SO_4^{2-} bound and glutamine bound CTPS from *T. thermophilus* suggests a model whereby upon ATP and UTP binding the glutamine amidotransferase domain and the amidoligase domains of CTPS come closer together (Figure 1.3) allowing passage of the liberated ammonia from the glutaminase reaction to the waiting 4-phosphoryl UTP in the amidoligase domain. In the structure of *T. thermophilus* CTPS (apo, SO_4^{2-} and glutamine bound) no ammonia tunnel was observed between the glutamine amidotransferase domain and the amidoligase domain. The authors support a model whereby ATP and UTP binding promote a conformational change involving the rotation of the glutamine amidotransferase domain 25° towards the amidoligase domain allowing a channel to form for ammonia propagation from the glutamine amidotransferase domain to the amidoligase domain (Goto et al., 2004). There are several pieces of evidence supporting and making this model plausible. One is that there is a flexible linker region separating the amidoligase domain from the glutamine amidotransferase domain permitting such movement. Another piece of evidence is that in the SO_4^{2-}-bound CTPS the glutamine amidotransferase domain is slightly rotated towards the amidoligase domain demonstrating

that there is flexibility in this direction. Further evidence supporting a conformational change is that without ATP and UTP and at high protein concentration, the apparent molecular weight as determined by dynamic light scattering was 470,000 and in the presence of ATP and UTP the apparent molecular weight was the more expected value of 250,000, suggesting that the ATP and UTP bound CTPS tetramer is more compact (Figure 1.3) than the unbound CTPS (Figure 1.2). The authors also noted that the residues proposed to be involved in this interaction between the two domains are disordered and/or highly mobile suggesting these residues could be involved in conformational changes of CTPS (Goto et al., 2004). As mentioned in the section regarding GTP binding, evidence from mutational analysis and sequence conservation suggest that GTP binding involves residues from both the glutamine amidotransferase domain and the amidoligase domain and upon the conformational change proposed by the authors, these residues would be in closer proximity to bind GTP (Goto et al., 2004). GTP binding would act as a stabilizing molecule promoting the more compact tetramer formation (Figure 1.3) and/or the formation of the ammonia tunnel, which may in part explain how GTP allosterically activates CTPS.

1.1.7 CTPS Regulation

1.1.7.1 Transcriptional Regulation

The only published transcriptional regulation of CTPS has been demonstrated in bacteria. CTPS transcription is regulated in bacteria, at least in gram positive bacteria, by the formation of a terminator hairpin structure in the mRNA leader sequence of CTPS under high levels of CTP and formation of an antiterminator hairpin in the leader sequences when CTP levels are low. The formation of the terminator hairpin and the antiterminator hairpin are

mutually exclusive and their formation is directly regulated by the availability of CTP. In the nascent mRNA leader sequence of bacteria CTPS there is the sequence 5'-gggc-3', where the polymerase will stutter (also known as reiterative transcription) if not enough CTP is available for adding the last "c" in this sequence. Instead of adding a C, extra G residues will be added until a C is finally added to continue with the transcription. The extra G's added will form the antiterminator hairpin structure at the exclusion of the formation of the terminator hairpin allowing transcription of CTPS to proceed. However, if CTP is readily available for the transcription of the last C in the 5'-gggc-3' sequence, the terminator hairpin structure will form and transcription of CTPS will be halted (Meng et al., 2004).

The transcription factor c-Myc has been shown to regulate other nucleotide metabolic enzymes (Mai and Jalava, 1994; Miltenberger et al., 1995; Schuhmacher et al., 2001), we therefore investigated CTPS protein expression in c-Myc $^{-/-}$ cells. CTPS protein, as assessed by immunoblot, was lower in c-Myc$^{-/-}$ cells than in c-Myc$^{+/+}$ cells. Re-addition of c-Myc (c-Myc adenovirus) into c-Myc$^{-/-}$ cells increased CTPS expression (Figure 4.12). The increase in CTPS1 expression in the c-Myc addback cells was further enhanced in the presence of serum, indicating that growth factor mediated CTPS1 protein expression may require c-Myc. Additionally, we have identified two canonical E-boxes (c-Myc binding sites), CACGTG, (Blackwell et al., 1990) at -70 and -300 base pairs (bps) upstream from the translation start site of human CTPS1. This evidence suggests that the transcription factor c-Myc may coordinately regulate the expression of CTPS with other pyrimidine metabolic enzymes (e.g. CAD) in mammalian cells (Eric M. Wauson and Matthew J. Higgins, unpublished observations).

1.1.7.2 Post-transcriptional Regulation

1.1.7.2.1 Translation Regulation

Translation regulation of CTPS has not been studied to date, but the mRNA for human CTPS1 contains a ~150 bp 5' UTR and an even larger ~900 bp 3' UTR giving ample sequence for regulatory proteins to bind and RNA secondary structures to form.

1.1.7.2.2 Regulation of CTPS by Phosphorylation

The study of CTPS phosphorylation has been studied extensively in the yeast *S. cerevisiae*. PKC was the first kinase shown to phosphorylate URA7. Comparison of PKC phosphorylation of URA7 *in vitro* with the phosphorylation of URA7 *in vivo* revealed that *in vitro* PKC phosphorylates URA7 on both serines and threonines, whereas URA7 from intact cells was found to be primarily phosphorylated on serine residues. Furthermore, separation of tryptic peptides by 2D-TLC from *in vitro* PKC-phosphorylated URA7 and *in vivo* phosphorylated URA7 revealed additional phosphorylated peptides from the *in vitro* phosphorylated URA7 than the *in vivo* phosphorylated URA7, indicating that, *in vitro*, PKC is phosphorylating sites not phosphorylated *in vivo* (Yang and Carman, 1995). Phosphorylation of URA7 by PKC *in vitro* increased the apparent V_{max} with respect to UTP under subsaturating ATP concentrations and the phosphorylated enzyme had a greater apparent affinity for ATP than the native enzyme. *In vitro* phosphorylation of URA7 by PKC also reduced feedback inhibition by CTP under subsaturating concentrations of ATP. PKC phosphorylation did not affect regulation of URA7 activity by glutamine and GTP, indicating that *in vitro* phosphorylation of URA7 by PKC increases CTPS activity under subsaturating conditions of ATP not by altering CTPS activity with respect to glutamine and

GTP, but by altering substrate affinity and the affinity for CTP feedback inhibition (Yang et al., 1996).

Possible PKC phosphorylation sites in URA7 were identified by using synthetic peptides derived from sequences in URA7 containing amino acid residues; S36, S330, S354, and S454 (*S. cerevisiae* numbering) (Park et al., 2003). However, when non-phosphorylatable mutants of full length URA7 were incubated *in vitro* with PKC, only the non-phosphorylatable, S330A, mutant had decreased phosphorylation suggesting this is the major PKC site in intact cells (Park et al., 2003). CTPS activity of these phosphorylation site mutants revealed that S36A and S354A had decreased CTPS activity, S330A caused an increase in activity and S454A had no significant effect on activity (Park et al., 2003). Interestingly, S330 is already an alanine in human CTPS1.

Additionally, this same group has identified *in vitro* S424 as a protein kinase A (PKA) phosphorylation site (Park et al., 1999). The non-phosphorylatable S424A mutant showed decreased CTPS activity and greater sensitivity to CTP feedback inhibition (Park et al., 1999). As was observed with in vitro phosphorylation of URA7 with PKC, PKA also stimulated URA7 activity (Yang and Carman, 1996). PKA stimulated URA 7 activity by increasing the V_{max} with respect to UTP, increasing both the V_{max} and the affinity for ATP and decreasing the ability of CTP to feedback inhibit URA7. As observed with PKC phosphorylation of URA7, the effects of PKA on URA7 activity were more apparent at subsaturating concentrations of ATP (Yang and Carman, 1996). Phosphorylation of S424 in URA7 by PKC and PKA was also shown to regulate phosphatidylcholine biosynthesis via the Kennedy pathway for phospholipid synthesis (Choi et al., 2003). Most recently

expression of human CTPS1 in *S. cerevisiae* showed that PKA and PKC could phosphorylate human CTPS1. Human CTPS1 was phosphorylated on Thr455 by both PKA and PKC whereas Ser462 was only phosphorylated by PKC (Chang et al., 2007; Choi and Carman, 2007; Han et al., 2005). Mutation of Thr455 to alanine increased the amount of CTP and increased CTPS activity in cells suggesting that phosphorylation of this residue decreases CTPS activity (Chang et al., 2007; Choi and Carman, 2007; Han et al., 2005). Mutation of S462 to alanine decreased CTPS activity suggesting that phosphorylation of S462 increases CTPS activity (Chang et al., 2007).

1.1.8 CTPS Isoforms

Unlike in prokaryotes where only one isoform of CTPS is found, two or more CTPS isoforms have been noted in studies involving eukaryotes. In humans two isoforms exist, CTPS1 and CTPS2. CTPS1 has been mapped to chromosome 1 (1p34) and CTPS2 has been mapped to chromosome X (Xp22). They share 74% sequence identity at the amino acid level and 44-55% identity with the two isoforms found in *S. cerevisiae*, URA7 and URA8 (Huang and Graves, 2003). Functional or expression differences between human CTPS1 and CTPS2 have not been investigated thus far. Since the sequence identity between human CTPS1 and CTPS2 is high and the sequence identity of the region important for tetramer formation and dimer formation is equally high (87% and 88%, respectively) it is likely that CTPS1 and CTPS2 could form heterodimers and/or heterotetramers and this may explain some of the complicated biochemical data that has been documented for CTPS in the last half century.

In *S. cerevisiae* both URA7 and URA8 can compensate for loss of one of the CTPS enzymes, but the combined loss of URA7 and URA8 is lethal. Based on observations such

as CTP concentration in null mutants, URA7 appears to be responsible for the majority of CTP synthesized *S. cerevisiae* (McDonough et al., 1995; Ozier-Kalogeropoulos et al., 1994).

1.2 Pyrimidine Nucleotide Biosynthesis

1.2.1 Salvage Synthesis of CTP

The salvage synthesis of CTP requires the import of cytidine into the cell and phosphorylation of cytidine to CTP (Figure 1.4). Nucleosides including cytidine are imported into cells by membrane transporters. These transporters can be divided into two groups; equilibrative nucleoside transporters (ENTs) and concentrative nucleoside transporters (CNTs). The ENTs do not use an ion gradient to import nucleosides, but rely on concentration gradients of nucleosides to drive transport. CNTs use an ion gradient, the sodium ion gradient generated by the sodium-potassium-ATPase, for transporting nucleosides. Cytidine is a substrate for both ENTs and CNTs. Of the ENTs, cytidine transport is thought to be primarily through ENT2 since the apparent affinity of cytidine is substantially higher for ENT2 than ENT1 (Ward et al., 2000).

Cytidine can also be imported by CNT1, CNT2 and CNT3. CNT1 is a pyrimidine preferring CNT and has an apparent affinity in the low micromolar range for cytidine (Pastor-Anglada et al., 2005). CNT3 has also been shown to transport cytidine with an apparent affinity in the low micromolar range as well (Damaraju et al., 2005). CNT2 has recently been shown to also transport cytidine (Nagai et al., 2006).

Once imported into the cell cytidine is "trapped" in the cell by being phosphorylated to CMP by uridine/cytidine kinase (EC 2. 7. 1. 48, UCK) (Orengo, 1969). The next step in

the salvage synthesis of CTP involves phosphorylating CMP to CDP by cytidylate kinase (EC 2. 7. 4. 14, CMP kinase) (Ruffner and Anderson, 1969). The final step in the salvage synthesis of CTP is catalyzed by nucleoside diphokinase (EC 2. 7. 4. 6, NDPK, also known as non-metastatic 23 (NM23)) (Berg and Joklik, 1954).

Because the cytidine levels in plasma from humans is around 0.7 μM (Traut, 1994), the salvage pathway is thought to not play as significant of a role in the synthesis of CTP compared to the *de novo* synthesis pathway of CTP.

1.2.2 *De novo* Synthesis of CTP

The rate-limiting reaction in the *de novo* synthesis of CTP is catalyzed by CTPS (EC 6. 3. 4. 2). The substrate, UTP, can be synthesized by either the salvage or *de novo* synthesis pathway for UTP. The salvage pathway for UTP synthesis parallels the same steps as the salvage pathway for CTP synthesis (Figure 1.4). The rate-limiting step in the *de novo* synthesis pathway for UTP is catalyzed by the multifunctional CAD protein (EC 2. 7. 2. 9, EC 2. 1. 3. 2 and EC 3. 5. 2. 3), which uses aspartate, bicarbonate, glutamine and ATP as substrates and is allosterically activated by PRPP. The product of the CAD reaction is converted to UMP through three more reactions, which is converted to UTP by the same kinases that convert UMP to UTP from the salvage pathway (reviewed in (Jones, 1980)).

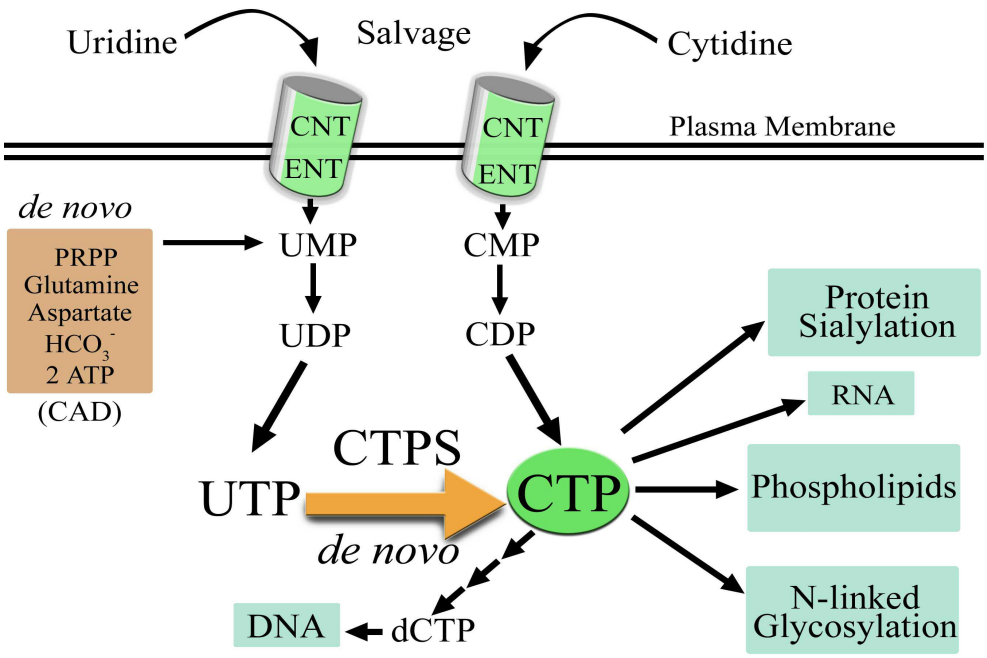


Figure 1.4 Salvage and *De novo* Pyrimidine Biosynthesis Pathways

Pyrimidines can be synthesized either by the salvage pathway, which requires import of nucleosides through the nucleoside transporters (ENTs or CNTs) and phosphorylation to the appropriate nucleotide, or the *de novo* pathway, which involves CAD and CTPS as the two rate-limiting enzymes for the synthesis of UTP and CTP, respectively. Also shown are some of the CTP-requiring processes (e.g. protein sialylation, DNA/RNA synthesis, phospholipid synthesis and N-linked glycosylation). PRPP is phosphoribosyl pyrophosphate.

1.3 Chemical Inhibitors and CTPS in Disease

1.3.1 Involvement in Cancer

Not only are CTP and dCTP levels elevated in a variety of cancers, but CTPS activity is also elevated in various cancers such as those derived from blood cells (leukemia), liver, colon and lung (de Korte et al., 1987a; De Korte et al., 1986; de Korte et al., 1987b; de Korte et al., 1985; Olah and Weber, 1979; Shewach, 1992; Sidi et al., 1985; van den Berg et al., 1993; van den Berg et al., 1994; van den Berg et al., 1995; Verschuur et al., 1998; Weber et al., 1980). Whether this increase in CTPS activity is due to elevated protein expression or due to mutations in CTPS rendering the enzyme more active have not been thoroughly investigated. Mutations rendering CTPS resistant to chemotherapeutic drugs as well as CTP feedback inhibition have been documented, suggesting loss of CTP feedback inhibition may be one mechanism by which CTPS activity is elevated (Blaney et al., 1993; Whelan et al., 1993; Whelan et al., 1994; Wylie et al., 1996; Zhang et al., 1993).

1.3.2 Involvement in Parasitic, Bacterial and Viral Infection

Many pathogens exert control over metabolic processes that assure their replication and survival. Since CTP levels are one of lowest concentration of NTPs in cells, control of CTP synthesis is vital to survival of many pathogens. Viruses need to replicate their genome for viral amplification and therefore require NTPs and/or dNTPs for pathogenesis. Several CTPS inhibitors alone or in combination therapy have demonstrated potent antiviral properties against viruses such as influenza, parainfluenza, coxsackie, sindbis, cytomegalo,

HIV, poxvirus, hemorrhagic fever and yellow fever (Andrei and De Clercq, 1993; De Clercq et al., 1991; Dereuddre-Bosquet et al., 2004; Gao et al., 2000; Neyts and De Clercq, 2003; Neyts et al., 1996).

African sleeping sickness is caused by trypanosomes (protozoans), which are spread to humans upon a bite by a tsetse fly. CTP pools in trypanosomes are low compared to other eukaryotic cells and trypanosomes do not have the ability to synthesize CTP via the salvage pathway using cytidine, but do encode their own CTPS (Hofer et al., 1998; Hofer et al., 2001). Trypanosome CTPS has a higher K_m for UTP than mammalian CTPS possibly explaining why CTP levels are lower in trypanosomes than in eukaryotic cells. Using the glutaminase inhibitor, acivicin, at a concentration below where CNS toxicity is observed, it was shown in mice that CTPS inhibition could suppress trypanosome infection demonstrating that targeting CTPS could be a new therapy for African sleeping sickness (Fijolek et al., 2007).

Other parasites and bacteria known to encode their own CTPS include *Plasmodium falciparum* (malaria), *Giardia intestinalis* and *Chlamydia trachomatis* (Hendriks et al., 1998; Jimenez and O'Sullivan, 1994; Tipples and McClarty, 1995). It may be possible to target the CTPS from these organisms as well as providing a new therapeutic strategy.

1.3.3 CTPS Inhibitors

1.3.3.1 The Rise and Fall of CPEC

As with many things throughout human civilization, good or bad, CPEC got its start from fermentation. In 1979, a novel carbocyclic nucleoside was discovered in a Japanese fermentation broth and named neplanocin A (Hayashi et al., 1980). Neplanocin A was quite

toxic and became a lead compound for synthesis of other carbocyclic nucleosides. Approximately 20 purine and pyrimidine analogs of neplanocin A were synthesized and evaluated for antitumor and antiviral properties (Arita et al., 1983). Cyclopentenyl cytosine, CPEC, was the most biologically active pyrimidine compound tested. CPEC was shown to be effective against a variety of tumor cells and tumor xenografts including colon carcinoma cells, leukemia cells, human lung cancer, melanoma and breast cancer cells (Glazer et al., 1986; Glazer et al., 1985; Marquez et al., 1988; Moyer et al., 1986). In 1989, the active metabolite of CPEC, CPEC triphosphate, was identified and CTPS was found to be the target (Kang et al., 1989). The pharmacokinetics of CPEC were studied thoroughly in nonhuman primates, dogs and rodents. In both rodents and dogs CPEC was primarily eliminated by renal excretion, but in nonhuman primates the primary route of elimination was by deaminating CPEC to cyclopentenyl uracil, CPEU (Blaney et al., 1990; Zaharko et al., 1991). Since CPEU inhibits uridine/cytidine kinase, UCK, it was thought that the antitumor effect of CPEC may be due to inhibition of UCK. However, testing revealed that not only is CPEU not as cytotoxic as CPEC, but pretreatment of cells with CPEU protected cells from the cytotoxic effects of CPEC (Blaney et al., 1992). Since there seemed to be an active cytidine deaminase converting CPEC to CPEU in primates, it was concluded that a continuous infusion of CPEC would be need for clinical trials. Phase I clinical trials commenced in 1994 with the continuous infusion of CPEC resulting in plasma levels of 0.4 to 3.1 μM CPEC, but the promise of CPEC as a new antitumor drug was thwarted when two people died of hypotension in the study (Politi et al., 1995). The exact reason for how or if CPEC caused hypotension is currently unknown. Perhaps if we understand more of how CTPS functions we can develop new therapies targeting CTPS without the devastating side-effects.

Although, CPEC is not currently being used clinically it is still a useful tool for studying the role of CTPS in *in vitro* cell systems.

1.3.3.2 Glutaminase Inhibitors

Glutaminase inhibitors such as acivicin and DON target the glutaminase reaction of CTPS. Although, many other glutaminase containing enzymes have the acivicin and DON targeted catalytic residues (catalytic cysteine of the catalytic triad), much of the antiproliferative effects of acivicin and DON are attributed to inhibition of CTPS as suggested by observations in leukemia cells (Fischer et al., 1988; Lyons et al., 1990). GMP synthase is the other glutaminase containing enzyme that acivicin inhibits almost as well as CTPS (Aoki et al., 1982; Weber et al., 1984). Acivicin works by alkylating the catalytic cysteine rendering the glutaminase nonfunctional (Cooney et al., 1976; Hanka and Dietz, 1973). It is possible the reason acivicin targets the glutaminase domain of CTPS better than other glutaminases is because the catalytic pocket is larger in CTPS than other glutaminases. Although, DON will also covalently inhibit the glutaminase reaction of CTPS, acivicin has a longer half life *in vivo* and penetrates the blood brain barrier better making it a better therapeutic drug in many instances (Chikhale et al., 1995; McGovren et al., 1982; Sullivan et al., 1988).

Another glutaminase inhibitor is glutamate γ-semialdehyde, GSA, which mimics the transition state of the glutaminase reaction and reversibly inhibits glutaminase activity (Bearne et al., 2001; Bearne and Wolfenden, 1995). GSA was shown to be a potent reversible inhibitor of CTPS (Bearne et al., 2001). Unfortunately, GSA exists in unfavorable equilibrium with pyrroline-5-carboxylate (P5C) a cyclic molecule, which does not have anti-

glutaminase activity. Interestingly, P5C is an intermediate in proline, glutamate and ornithine metabolism (review see (Jones, 1983; Jones, 1985)) suggesting that under conditions where these intermediates accumulate and/or are released, GSA could form and inhibit CTPS.

1.3.3.3 Other CTPS Inhibitors

3-deazauridine (DAU), when phosphorylated to the triphosphate form, competes with UTP for binding to CTPS and thereby inhibiting CTP biosynthesis (McPartland et al., 1974; Van Kuilenburg et al., 1994). Additionally, DAU in combination with cytosine arabinoside (AraC) was shown to have synergistic effects in leukemia cells (Momparler et al., 1991).

1.4 CTP Dependent/Requiring Processes

1.4.1 DNA/RNA Biosynthesis

dCTP and CTP are essential for synthesis of DNA and RNA, respectively. Whether pools of nucleotides exist for the formation of DNA/RNA or other nucleotide conjugated products has been suggested. For instance, using rat hepatocytes labeled with [^{3}H]uridine [^{3}H]cytidine and/or [^{14}C]orotic acid (intermediate in the UTP *de novo* synthesis pathway) it was suggested that salvage synthesized cytidine nucleotides were more preferentially used for RNA synthesis and synthesis of cytidine compounds (Pels Rijcken et al., 1993). Another study using a similar strategy of labeling cells with radiolabeled precursors found in undifferentiated PC12 cells that the salvage pathway of CTP synthesis seemed to be more preferred for RNA synthesis (Slingerland et al., 1995). Investigation of the pools of dCTP involved in DNA repair has found that the import of deoxycytidine is preferentially used for

the DNA repair in response to alkylating DNA damaging agents and radiation, whereas intracellular stores of dCTP are probably preferentially used for replicative DNA synthesis (Xu et al., 1995). Based on multiple studies, some of which are mentioned above, it seems that there is a compartmentation of nucleotides involved in DNA/RNA synthesis, and possibly other nucleotide requiring process, but evidence directly demonstrating such pools exist is lacking.

1.4.2 CTP Involvement in Thymidine Metabolism

Interestingly, an unexpected link between CTP metabolism and dTTP formation involving CTPS has been elucidated. CHO cells selected for resistance to AraC required exogenous thymidine, deoxycytidine or deoxyuridine for survival and levels of dCTP were found to be elevated (Mark Meuth, 1979). Further investigation revealed elevated CTP and dCDP levels, but uridine nucleotide levels remained unchanged suggesting that cytidine nucleotides are involved in thymidine auxotrophy. Additionally, CTP was demonstrated to inhibit reduction of UDP to dUDP, which through several reactions is converted to dTTP (de Saint Vincent et al., 1980). It was later shown in thymidine auxotrophic cell lines that CTPS was less sensitive to CTP feedback inhibition than the revertant cells and this was most likely the cause for the elevated CTP levels and the AraC resistance originally observed (Trudel et al., 1984). The model proposed for how increased CTP levels can cause thymidine auxotrophy is that increased CTP levels leads to increased CDP, which, along with UDP, is a substrate for ribonucleotide reductase and since ribonucleotide reductase has a higher affinity for CDP than UDP, UDP is not reduced to dUDP to form dTTP resulting in thymidine auxotrophy (Figure 1.4) (Eriksson et al., 1979; Trudel et al., 1984). Analysis of DNA from

CTPS resistant to various drugs including AraC revealed a clustering of mutations proposed and confirmed later by structural studies to be in the CTP binding pocket (Whelan et al., 1993). The ability of CTP levels to impact dTTP formation demonstrates a possible role for CTP in regulating the balance of dNTPs needed for DNA synthesis.

1.4.3 Phospholipid Biosynthesis

Phospholipids are not only structural molecules forming membranes around cellular structures, but are also involved in signaling through the formation of docking sites for proteins and generating second messengers. CTP is required for phospholipid synthesis because it is used to form the energy rich intermediates involved in phospholipid synthesis. Elevated CTP levels can increase the flux through the rate-limiting enzyme involved in phosphatidylcholine biosynthesis most likely resulting in increased phosphatidylcholine biosynthesis (Choy et al., 1980). Most phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are synthesized via the Kennedy pathway, where phosphocholine and phosphoethanolamine react with CTP to form CDP-choline and CDP-ethanolamine. CDP-choline and CDP-ethanolamine then react with diacylglycerol to form PC and PE, respectively (Figure 1.5) (Kennedy, 1956a; Kennedy, 1956b; Kennedy and Weiss, 1956). Phosphatidylinositol (PI) and cardiolipin also require CTP for synthesis as they are synthesized from the intermediate CDP-diacylglycerol (CDP-DAG) (Figure 1.5) (Benjamins and Agranoff, 1969; Hostetler et al., 1971; Paulus and Kennedy, 1960; Prottey and Hawthorne, 1967). In mammals phosphatidylserine (PS) is synthesized by a base exchange reaction of serine with either PC or PE, whereas in yeast PS is synthesized from

CDP-DAG and serine (Figure 1.5) (Borkenhagen et al., 1961; Kuge et al., 1986a; Kuge et al., 1986b).

Inhibition of CTPS with CPEC decreased PC, PE, PI, PS and cardiolipin levels in H9c2 cardiac myoblasts (Hatch and McClarty, 1996). Interestingly, neutral lipids were slightly increased in the presence of CPEC suggesting inhibition of phospholipid synthesis results in increases in neutral lipid formation.

1.4.4 Protein Sialylation

Sialic acids are comprised of over 50 related sugars derived from neuraminic acid (5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranos-1-onic acid) or deaminoneuraminic acid (KDN, 3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranos-1-onic acid). The sialylation proteins is important in a variety biological processes, such as cell-cell communication (for review (Crocker et al., 2007; Munday et al., 1999; Varki, 2007). Sialic acids are activated before they are transferred to proteins by reacting with CTP to form CMP-sialic acid. Incubation of cells with the CTPS inhibitor, DAU, can inhibit the regeneration of surface sialic acid containing proteins demonstrating that CTP levels can influence plasma membrane expression of sialylated proteins (Hindenburg et al., 1985).

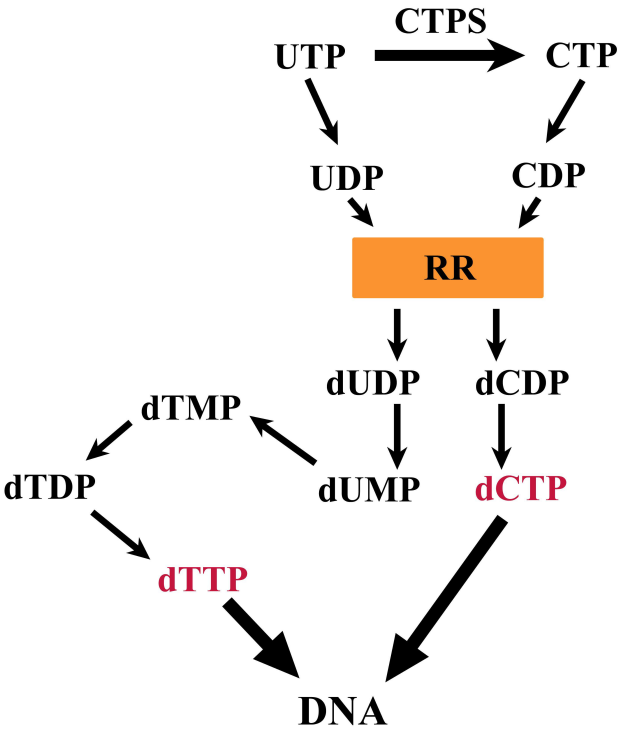


Figure 1.5 dTTP Biosynthesis Pathway

Both UDP and CDP are substrates for ribonucleotide reductase (RR), but CDP has a two fold higher affinity for RR. Under elevated CTP and CDP conditions CDP is more readily reduced at the expense of reducing UDP and thought to cause decreased levels of dTTP.

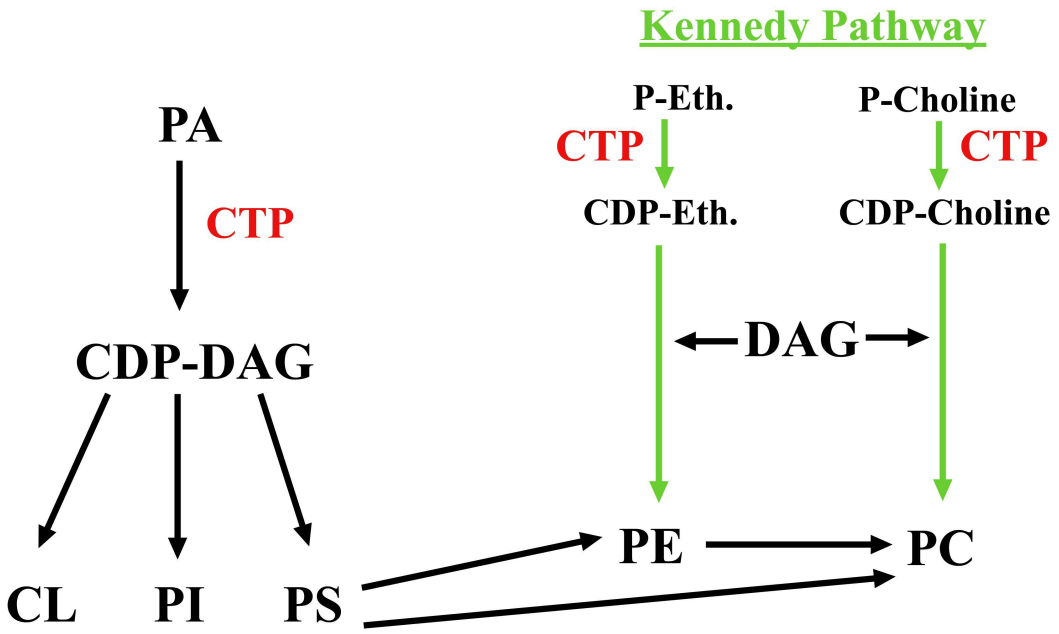


Figure 1.6 Phospholipid Synthesis Pathway

Biosynthesis of phospholipids requires CTP. Shown is the Kennedy pathway for the synthesis of phosphatidylcholine (PC) and phosphatidylethanolamine (PE). PC and PE can also be synthesized from phosphatidylserine (PS). In addition to being required for the Kennedy pathway, CTP is required for the formation of CDP-diacylglycerol (CDP-DAG) from phosphatidic acid (PA). CDP-DAG is used in the synthesis of cardiolipin (CL), phosphatidylinositol (PI) and PS.

1.4.5 N-linked Glycosylation

Attachment of oligosaccharides to asparagine residues of proteins requires that these oligosaccharides are assembled on dolichyl (dolichol) phosphate, which is formed by dolichol kinase using CTP and not ATP or GTP as the phosphoryl donor for the phosphorylation (Burton et al., 1979). Thus, this is another CTP-dependent process. The primary means of regulating the involvement of dolichol in N-linked glycosylation is by phosphorylation suggesting CTP may play an important role in regulating N-linked glycosylation (for review see (Rip et al., 1985)).

1.5 Role of GSK3 in Metabolism Regulation

GSK3 was originally identified as the third kinase responsible for the phosphorylation of glycogen synthase, which decreased glycogen synthase activity (Embi et al., 1980; Rylatt et al., 1980). 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 (Frame and Cohen, 2001)). Interestingly, the carboxy-terminus of mammalian CTP: phosphocholine cytidylyl transferase (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 (MacDonald and Kent, 1994; Watkins and Kent, 1991). Moreover CCT activity is decreased by phosphorylation (MacDonald and Kent, 1994; Watkins and Kent, 1991). Multiple phosphorylation sites in the carboxy-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 possibly CTPS1.

1.6 Specific Aims

Maintaining proper metabolic control of key metabolic pathways is critical to sustaining homeostasis. Misregulation, either through genetic mutation or infection by a pathogens, of key regulatory enzymes can contribute to diseases such as cancer. CTP is an important nucleotide involved in processes including protein sialylation, phospholipid synthesis, DNA/RNA synthesis and N-linked glycosylation. CTP concentrations in mammalian cells are tightly controlled primarily by the enzyme CTPS. CTPS can bind four nucleotides and glutamine making CTPS sensitive to and exquisitely regulated by metabolites. In *S. cerevisiae*, URA7 activity was shown to be regulated by PKA and PKC phosphorylation. Furthermore, phosphorylation of URA7 was shown to impact phospholipid synthesis.

The goal of this dissertation was to determine how mammalian CTPS1 was regulated by phosphorylation and to determine CTPS1 interacting proteins, which may aid in further determining how mammalian CTPS1 is regulated. For both of these aims, a human/rodent specific CTPS1 antibody was developed. Using hCTPS1 from [^{32}P]-orthophosphate labeled HEK 293 cells that were treated with activators and inhibitors of interest. Since URA 7 was phosphorylated by PKA and PKC, the ability of hCTPS1 to be phosphorylated by PKA and/or PKC was first investigated (specific aim 1). Determination of phosphorylation sites in endogenous hCTPS1 was done by mass spectroscopy and verified using phospho-null

mutants of the identified phosphorylation sites expressed in intact HEK 293 cells (specific aim 2). Since many of the phosphorylation sites identified in yeast were not conserved in human CTPS1, stimuli that regulated hCTPS1 phosphorylation was also investigated by immunoprecipitating hCTPS1 from [^{32}P]-orthophosphate labeled HEK 293 cells treated with various factors (specific aim 3).

In addition to determining how hCTPS1 is regulated by phosphorylation, hCTPS1 interacting proteins were identified by mass spectrometry and one was verified by immunoprecipitation and reciprocal immunoprecipitation (specific aim 4). Thus, these studies provide a comprehensive analysis of the factors that regulate CTPS activity in mammalian cells.

Specific Aims:

(1) Determine if PKA and/or PKC phosphorylate endogenous hCTPS1 (Chapter 3)

(2) Determine on what residues hCTPS1 is phosphorylated (Chapter 3)

(3) Determine what stimuli and/or kinases other than PKA and PKC phosphorylate CTPS1 (Chapter 3)

(4) Determine hCTPS1 interacting proteins (Chapter 4)

CHAPTER

2. Experimental Procedures

2.1 Materials

Taxol and anti-α-tubulin antibody was purchased from Sigma. CPEC was acquired from NCI (National Cancer Institute). GST-xPin1 was a kind gift from Dr. Anthony Means (Duke Univesity). c-Myc$^{-/-}$ MEFs, c-myc$^{+/+}$ MEFs and c-myc adenovirus were kind gifts from Dr. Phil Nevins (Duke University).

2.2 CTPS Activity Assay

CTPS1 was immunoprecipitated as described above. Immunoprecipitates were washed three times with 0.5 ml RIPA buffer without SDS containing 5 µg/ml leupeptin and a final wash was done with 1 ml of 1 X CTPS reaction buffer (50 mM Tricine, pH8.1, 20 mM $MgCl_2$ and 5 mM KCl). The volume was adjusted to 40 µl with 10 µl 5X reaction buffer and 30 µl ddH_2O. CTPS1 and 5X substrate mix (5 mM ATP, 0.5 mM UTP, 5 mM GTP, 100mM L-glutamine and 0.625 µCi/ml [^{14}C]-UTP or 0.1 µCi/ml [^{3}H]-UTP) were equilibrated to 37ºC. The reaction was initiated by adding 10 µl of 5X substrate mix to 40 µl of CTPS1 in reaction buffer and stopped after 45 minutes by adding 10 µl 200 mM EDTA and removing tube to ice. CTPS1 reactions were reduced to dryness using a SpeedVac. Dried supernatant pellets were resuspended in 10 µl of ddH_2O. 3-4 µl of resuspended pellets and 1 µl of 100 mM UTP/CTP/UDP/CDP standards were spotted onto polyethylimine (PEI) cellulose plastic backed plates (Fisher Scientific) that were pre-washed 10 minutes with

ddH_20, 5 minutes with 0.65 N HCl and 1 minute 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 with a short wavelength UV light and nucleotide areas containing radioactivity were visualized by exposing TLC plate to phosphorimage screen overnight. Areas corresponding to UTP, CTP, UDP and CDP were excised and counted using Ecoscint™ (Fisher Scientific) scintillation fluid.

2.3 Transfection of HEK 293 cells

75% confluent 100 mm plate were transfected with 5.6 µg DNA using Lipofectamine™ as outlined by manufacturer. Briefly, 5.6 µg DNA and 22.5 µl Lipofectamine™ were diluted with 560 µl DMEM. Diluted DNA and Lipofectamine™ solutions will be combined and allowed to incubate at room temperature for 30 minutes. Following 30 minutes, 3.8 ml DMEM was added to each DNA/Lipofectamine™ solution and the newly diluted DNA/Lipofectamine™ solution is added to a plate containing HEK 293 cells. After plate has incubated for 5 hours at 37ºC/5% CO_2, FBS/penicillin/streptomycin in DMEM is added to achieve a final concentration of 10% FBS/ 100 U/mL penicillin / 100 µg/mL streptomycin.

2.4 Transfection of HELA cells

$1X10^6$ HELA cells in a 100 mm plate was transfected with 7 µg DNA using Lipofectamine™. 7 µg DNA and 60 µl Lipofectamine™ was diluted with 800 µl Optimem™. DNA and Lipofectamine™ solutions will be combined and allowed to incubate at room temperature for 30 minutes. DNA/ Lipofectamine™ solution will be diluted with 6.4 ml Optimem™ and layered onto HELA cells. Following incubation of HELA cells for 5

hours at 37ºC/5% CO_2, transfection media will be removed and HELA cells will be washed with DMEM/10% FBS / 100 U/mL penicillin / 100 µg/mL streptomycin and placed in fresh DMEM/10% FBS 100U/mL penicillin /100 ug/mL streptomycin.

2.5 Immunostaining

Cells were plated onto glass coverslips. Following treatment and/or transfection glass coverslips were removed and fixed in 4% p-formaldehyde/4% sucrose in phosphate buffered saline (PBS) for 10 minutes. Fixing reaction was quenched with 10 mM glycine / 1% Triton X-100 in PBS. After washing coverslips three times with PBS with 1% Triton X-100 (PBST), coverslips were placed in blocking solution consisting of 5% BSA/ 5% horse serum in PBST for at least four hours. Blocking solution was removed and ~35 µl of the primary antibody diluted in blocking solution will be added to each coverslip. Coverslips were incubated in a humidifying chambers for 4 hours at room temperature to overnight at 4ºC. Following incubation with primary antibodies, coverslips were washed with PBST. Secondary antibodies containing either Alexa 488™ or Alexa 594™ fluorophore was added to coverslips and incubated at room temperature in the dark for 1 hour. Coverslips were removed and washed 3 times with PBST. Washed coverslips were mounted onto glass slides with Permafluor™ and sealed with finger nail polish.

2.6 Antibodies and immunoblotting.

Anti-CTPS1 (human) antibody was generated by Rockland Immunochemicals using a C-terminal peptide (amino acids 578-591, H_2N-CSEITELKFPSINHD-COOH) containing an N-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 4ºC by adding ice-cold saturated $(NH_4)_2SO_4$ to a final percentage of 50%. $(NH_4)_2SO_4$ precipitated proteins were resuspended in a volume of PBS equal to the initial volume of serum and dialyzed overnight at 4ºC against PBS. Second, 2.5 ml of $(NH_4)_2SO_4$ purified serum protein solution was rotated with 0.5 ml (bead volume) protein A that has been equilibrated with 100 mM Tris pH 8.0. After washing the protein A column with 100 mM Tris pH 8.0 and 10 mM Tris pH 8.0, bound antibodies were eluted with 5 ml of 50 mM glycine pH 3.0. One ml fractions were collected in microfuge tubes containing 100 µl 1 M Tris pH 8.0 to immediately neutralize pH. All fractions were pooled and dialyzed overnight at 4ºC against PBS. Lastly, antibodies specific for CTPS1 were separated from non-specific antibodies using a peptide affinity column created with the antigenic peptide (H_2N-CSEITELKFPSINHD-COOH) and a Sulfolink column (Pierce Biotechnologies) per manufacturers instructions. Eluted anti-CTPS1 antibodies were concentrated to about 350 µl and buffer exchanged to PBS with 0.05% NaN_3 using 4 ml 10 kDa MWCO spin concentrators. Twenty µl aliquots were stored at -20ºC for future use.

Immunoblotting for GSK3 (Santa Cruz) and phospho-GSK3 (Cell Signaling) (α/β, Ser21/Ser9) was done by lysing HEK 293 cells with RIPA without SDS and determining the protein concentration in lysate using the method of Bradford (Bradford, 1976). Lysates were prepared for SDS PAGE by adding an equal volume of 2X SDS sample buffer (0.5 M Tris pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.002 g/ml bromophenol blue). 10 to 40 µg of protein was separated on an 8 or 10% discontinuous buffer gel and proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Immobilin-P, Millipore). Membranes were blocked for one hour at room temperature or

overnight in 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 (Ser9) 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 hour at room temperature (α-GSK3β) or overnight at 4°C (α-phospho (Ser9) GSK3β, Cell Signaling). Membranes were then washed with TBST three times five minutes each wash and incubated for one hour with 1:2000 goat anti rabbit (Santa Cruz) conjugated to horseradish peroxidase (for α-GSK3β) or alkaline phosphatase (for α-phopho (Ser9) GSK3β). Membranes were washed three times five minutes each wash with TBST and either visualized using enhanced chemiluminescent kit (Amersham) per manufacturers instructions and exposing membrane to X-ray film or using BCIP/NBT kit (Promega) to visualize alkaline phosphatase conjugated secondary antibody as suggested by manufacturer.

2.7 Preparation of Peptides from Excised Bands for Mass Spectrometry

Protein bands excised from Coomassie blue stained gels will be cut into small pieces and destained with 50 mM ammonium bicarbonate/ 50% acetonitrile solution. After gel pieces have been destained they was dried down using a SpeedVac. Gel pieces will be reswelled with 0.2 µg of trypsin and incubated overnight at room temperature. Supernatent was removed to a new tube and peptides were further extracted with 60% acetonitrile/5% formic acid. Volume of collected peptides were reduced to 10-15 µl using the SpeedVac.

2.8 Phosphorylation site identification by mass spectrometry.

HEK 293 cells were labeled and immunecomplexes 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) per manufacturer instructions. Bands corresponding to CTPS1 were excised and cut into about 1 mm pieces. Membrane pieces were placed in a siliconized microfuge tube and MemCode destaining was done as recommended by manufacturer. After washing membrane pieces with ddH_2O three times they were incubated with 1% w/v polyvinylpyrolidone (PVP) suspended in 100 mM acetic acid for at least one hour at 37ºC. Membrane pieces were washed four times with ddH_2O to ensure residual PVP was removed. Membrane pieces were incubated with 0.5 µg TPCK modified sequencing grade trypsin (Promega) in 25 mM ammonium bicarbonate pH 8.0 overnight at 37ºC. Peptides were collected by removing trypsin solution to a clean tube and the membrane pieces three times were washed with ddH_2O and removing each ddH_2O wash to the same clean tube containing the trypsin solution. The volume of collected peptides was reduced to a final volume of about 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 ddH_2O to remove ammonium bicarbonate. After the final ddH_2O 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 about 8 µl. The peptides were spotted in a corner of a 10x10 cm cellulose TLC plate, 1.5 cm from the sides of the plate. Peptides were electrophoresed using a Hunter thin layer electrophoresis box at 1000 V for 32 minutes. 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) (Scheidtmann et al., 1982). After exposure of TLC plates to X-ray film or phosphorimager plate radioactive spots indicative of phosphopeptides were eluted as described previously (Raska et al., 2002). Volume of peptides was decreased to about 10 µl using a speed vac. Peptides were subjected to nanospray electrospray ionization mass spectrometry (ESI-MS) on an Applied Biosystems QSTAR® pulsar mass spectrometer in positive ion mode. Peptides were sequenced by ESI-MS/MS using BioAnalyst software.

2.9 Cell Cycle Synchronization of HELA Cells

HELA cells were synchronized as described in (Erkmann et al., 2005).

2.10 In vivo labeling with [^{32}P]orthophosphate.

100 mm tissue culture plates (Sarstedt Inc.) were coated with 0.02 mg/ml poly-D-lysine (Sigma Aldrich) and washed with ddH_2O. 7.5 X 10^6 HEK 293 cells were plated on PDL coated plates. Cells were washed one time with 5 ml phosphate-free DMEM (phosphate/pyruvate free DMEM (Invitrogen Inc.) supplemented with sodium pyruvate (Invitrogen Inc.) giving a final concentration of 110 mg/L sodium pyruvate and the same concentration of penicillin/streptomycin as described earlier). Three milliliters of phosphate-free DMEM containing 10% dialyzed FBS (Invitrogen Inc.) was added to plates 30 minutes prior to adding 1 mCi of [^{32}P]-orthophosphate per plate (0.33 mCi/ml, MP Biomedical). In a typical experiment, cells were labeled for 4 hours prior to manipulation. Labeling was stopped by washing plates twice with 5 ml 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. Immunecomplex proteins were eluted from beads by boiling for 3 minutes with Laemmli buffer. SDS-PAGE/autoradiography was done on eluted proteins.

2.11 Calf Intestinal phosphatase treatment of CTPS1

Immunoprecipitated CTPS1 (prepared as described earlier) was washed three times with one milliliter RIPA buffer without SDS and one time with one milliliter 1X CTPS reaction buffer. 10 µl 5X CTPS reaction buffer and 25 µl ddH_2O was added to immunoprecipitates and tubes were placed at 30°C for phosphatase reaction. Dephosphorylation was initiated by adding 15 µl (1 unit/µl) calf intestinal phosphatase (CIP, Roch Biochemicals) or 15 µl 50% glycerol (control reaction) to tubes containing immunoprecipitated CTPS1 (50 µl total volume). Reactions were stopped by adding one milliliter ice-cold 1X CTPS reaction buffer to dilute reaction components and placing tubes on ice. Immunoprecipitates were washed again with ice-cold 1X CTPS reaction buffer.

2.12 GSK3β RNA interference.

3.5 X 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./strep.) was removed from cells and media was replaced with DMEM containing 10% FBS and no antibiotics (DMEM + 10% FBS). RNA duplexes (Dharmacon) were suspended as suggested by manufacturer and 15 µl of 20 µM RNA duplex was added to one tube containing 285 µl DMEM + 10% FBS and mixed thoroughly. 12 µl of Dharmafect 1 was added to a second tube containing 288 µl DMEM + 10% FBS and mixed thoroughly.

Tubes containing RNA duplexes and Dharmafect 1 were combined, mixed thoroughly and allowed to stand at room temperature at 20 minutes. 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 media removed. Media was switched the following day to DMEM + 10% FBS + pen./strep. Cellular manipulations were done the following day after incubating cells overnight.

2.13 Pin1 RNA interference

Silencing of Pin1 was done as described for the silencing of GSK3β (Experimental Procedures).

2.14 In vitro GSK3β kinase reaction.

Endogenous CTPS1 or FLAG-CTPS1 was immunoprecipitated as described above. Immunoprecipitates were washed one time with one milliliter 1X GSK3 reaction buffer (20 mM Tris pH 7.5 at 25°C and 10 mM $MgCl_2$) without DTT and 25 µl complete GSK3 reaction buffer (20 mM Tris pH 7.5 at 25°C, 10 mM $MgCl_2$, 10 µM ATP, 2 µl [^{32}P]-γ-ATP (10 mCi/ml, PerkinElmer), 5 µg/ml leupeptin, 10 nM microcystin and 5 mM DTT) was added to each immunoprecipitate. To each reaction 0.01 units of GSK3β purified enzyme (New England Biolabs) was added and incubated at 30°C for 30 minutes with flicking tubes occasionally to resuspend immunoprecipitates. Reactions were stopped by removing supernatant, placing tubes on ice and adding 30 µl of 1X SDS sample buffer (See immunoblotting for composition of SDS sample buffer) to immunoprecipitates.

2.15 Plasmid construction and site-directed mutagenesis.

Full length cDNA containing human CTPS1 was purchased from Invitrogen Inc. (IMAGE clone ID: 3355881). A C-terminal FLAG sequence was introduced after insertion of human CTPS1 into pCDNA 4 myc/his using the compatible cohesive ends Eag1&Not1 and Xho1. Briefly, the CTPS1 IMAGE clone was digested with Eag1 and Xho1 and ligated into pCDNA4 digested with Not1 and Xho1. Introduction of the C-terminal FLAG sequence was done by first amplifying a C-terminal section of CTPS1 with the sense primer 5'-CACGAAGCTTGGCAGAAGC-3' (HindIII site is underlined) and antisense primer 5'-GACTCGAGCGCTTGTCGTCATCGTCTTTGTAA TCCGCGTCATGATTTATTGATGG-3' (Xho1 site is underlined, FLAG sequence (DYKDDDDK) is boldface and mutation of stop codon to alanine is italicized & underlined) and finally ligating the C-terminal amplified section back into the pCDNA4 CTPS1 using HindIII and Xho1 restriction sites. A Kozak sequence (GCCACC) was added upstream of the start codon (ATG) by amplifying an N-terminal fragment of CTPS1 with the sense primer 5'-CGCGGCCGCGCCACCATGAAGTACATTCTG-3' (Not1 site is underlined and start codon is boldface) and antisense primer 5'-CGGATCCGCGTCATGATTTATTGATGG-3', digesting the amplified N-terminal fragment with Not1 and Eco N1 and ligating the digested N-terminal fragment into pCDNA4 myc/his CTPS1-FLAG digested with Not1 and Eco N1. Site-directed mutagenesis was done using Quickchange™ (Stratagene) per manufacturers instructions. All mutations were verified by sequencing. Of note, S571 was found to be an isoleucine in the IMAGE clone and was mutated back to the wild type serine.

2.16 c-Myc Adenovirus Infection

c-Myc adenovirus was added to MEFs in media without serum, to give a final concentration of 100 MOI. Expression was allowed to proceed from 24 hours and then media was replaced with media containing 10% FBS or 0.1% FBS.

CHAPTER

3. Phosphorylation Regulation of Human CTPS1

3.1 Abstract

Cytidine triphosphate synthetase (CTPS) catalyzes the rate-limiting step in the *de novo* synthesis of CTP and both the yeast and human enzymes have been reported to be regulated by PKA or PKC phosphorylation. Here, we provide evidence that stimulation or inhibition of PKA or PKC does not alter the phosphorylation of endogenous human CTPS1 in HEK 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 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}P]-orthophosphate labeled cells and analyzing the phosphopeptides by mass spectrometry identified S574 and S575 as phosphorylated residues. Mutation of S571 demonstrated that S571 was the major site phosphorylated by GSK3 in intact HEK 293 cells by GSK3 *in vitro*. Furthermore, mutation of S575 prevented the phosphorylation of S571 suggesting that phosphorylation of S575 was necessary for priming the GSK3 phosphorylation S571. 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 alkaline phosphatase increased CTPS1 activity in a time-dependent manner demonstrating that phosphorylation inhibits CTPS1 activity. Since many

other phosphorylation sites in the carboxyl terminus of hCTPS1 are CK I consensus phosphorylation sites and GSK3 and CK I can coordinately phosphorylate other substrates (e.g. β-catenin), we investigated whether CK I was also involved in hCTPS1 phosphorylation. Indeed, using the CK I inhibitor, D4476, CK I was found to be involved in hCTPS1 phosphorylation and contributed to the phosphorylation of multiple sites in the carboxy terminus of hCTPS1. This is the first study to investigate the phosphorylation and regulation of human CTPS1 in human cells and suggests that GSK3 is a novel regulator of CTPS activity and CK I may also be involved in regulating human CTPS1 function.

3.2 Introduction

Pyrimidine nucleotides have been known for decades to be essential components of cells. Most recently the effects of nucleotides have been revisited and were shown to regulate apoptosome formation by binding to cytochrome C and constitutively high levels of nucleotides were shown to inhibit cell cycle progression and the response to DNA damage (Chabes and Stillman, 2007; Chandra et al., 2006). Of all the nucleotides, the cellular concentration of cytidine triphosphate (CTP) is the lowest, suggesting that control of CTP synthesis is tightly regulated (Traut, 1994). CTP is important in DNA/RNA synthesis, phospholipid synthesis, protein sialylation and N-linked glycosylation, 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 (de Korte et al., 1987a; De Korte et al., 1986; de Korte et al., 1987b; de Korte et al., 1985; Harmenberg et al., 1990; Shewach, 1992; Sidi et al., 1985). 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 (van den Berg et al., 1993; van den Berg et al., 1994). Primary human lymphocytes demonstrate a dramatic increase in CTP levels following mitogenic stimulation, demonstrating the importance of CTP synthesis in response to growth stimuli (van den Berg et al., 1994).

Cytidine triphosphate synthetase (CTPS; EC: 6.3.4.2) catalyzes the rate-limiting step in the *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. 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 (Discussed in more detail in Chapter 1).

Regulation of yeast CTPS by phosphorylation was discovered by Carman and colleagues who showed that yeast CTPS1 (URA7) was phosphorylated *in vitro* on multiple sites by protein kinase C (PKC) (S36, S330, S354, and S454) (Park et al., 2003). Mutation of potential phosphorylation sites demonstrated that S330 was a major site of PKC phosphorylation (Park et al., 2003). Additionally, they identified S424 as an *in vitro* protein kinase A (PKA) phosphorylation site (Park et al., 1999). The S424A mutant showed decreased CTPS activity and greater sensitivity to CTP feedback inhibition (Park et al., 1999). Recently this same group also showed that human CTPS1 expressed in yeast was phosphorylated by PKA (Han et al., 2005). However, sequence alignment 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 (Figure

3.1), 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.

3.3 Results

3.3.1 PKA and PKC are not Involved in the Phosphorylation of hCTPS1 in Human Cells

CTPS from *S. cerevisiae* (URA7) was shown to be phosphorylated by PKA and PKC. However, amino acid sequence alignment of the phosphorylated residues in URA7 with the corresponding residues of human and mouse CTPS1 and CTPS2, showed that only two of the five residues are conserved in mammals (Figure 3.1). If PKA and/or PKC was involved in phosphorylating endogenous hCTPS1 in HEK 293 cells, the phosphorylation site would most likely not correspond to the sites identified in *S. cerevisiae* given that many of these sites are not conserved in mammals. To test whether PKA or PKC are involved in phosphorylation of endogenous hCTPS1, HEK 293 cells were labeled with [^{32}P]orthophosphate and then PKA or PKC were activated or inhibited. Activation of PKA by the cAMP elevating agent, forskolin, alone or in combination with the phosphodiesterase inhibitor (PDE) IBMX did not alter the phosphorylation of hCTPS1 despite the activation of PKA as assessed by in vitro kinase assay using KEMPtide as a substrate (Figure 3.2). Inhibition of PKA with up to 20 μM of the PKA inhibitor, H89, also did not alter hCTPS1 phosphorylation in HEK 293 cells (Figure 3.2). PKC was activated by the addition of the diacylglycerol analog PMA, which activated

PKC resulting in the increase in phosphorylation of ERK, but did not alter the phosphorylation of hCTPS1 in HEK 293 cells (Figure 3.3).

Inhibition of PKC with 10 μM Gö6983, which inhibits several PKC isozymes (Gschwendt et al., 1996; Stempka et al., 1997; Wang et al., 1998), also did not change the phosphorylation of endogenous hCTPS1 in HEK 293 cells (Figure 3.3). These results suggest that PKA and PKC are not involved in the phosphorylation of endogenous hCTPS1 in HEK293 cells under the conditions tested. While PKA and/or PKC may contribute to the phosphorylation of minor sites in hCTPS1 our results do not support a major role for these kinases in phosphorylating CTPS1.

3.3.2 GSK3 Regulation of Human CTPS1

3.3.2.1 Low-serum induces phosphorylation of endogenous hCTPS1 in a GSK3 dependent manner

Since inhibition or activation of PKA or PKC did not alter the phosphorylation of endogenous human CTPS1 from [^{32}P]orthophosphate labeled HEK 293 cells (Figure 3.2 and 3.3) we investigated other conditions that could affect CTPS phosphorylation (Table 3.1). In our search of stimuli and kinases that influence phosphorylation of endogenous hCTPS1 in HEK 293 cells, 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 [^{32}P]orthophosphate labeled HEK 293 cells in 0.1% FBS was approximately twice that from cells maintained in 10% FBS.

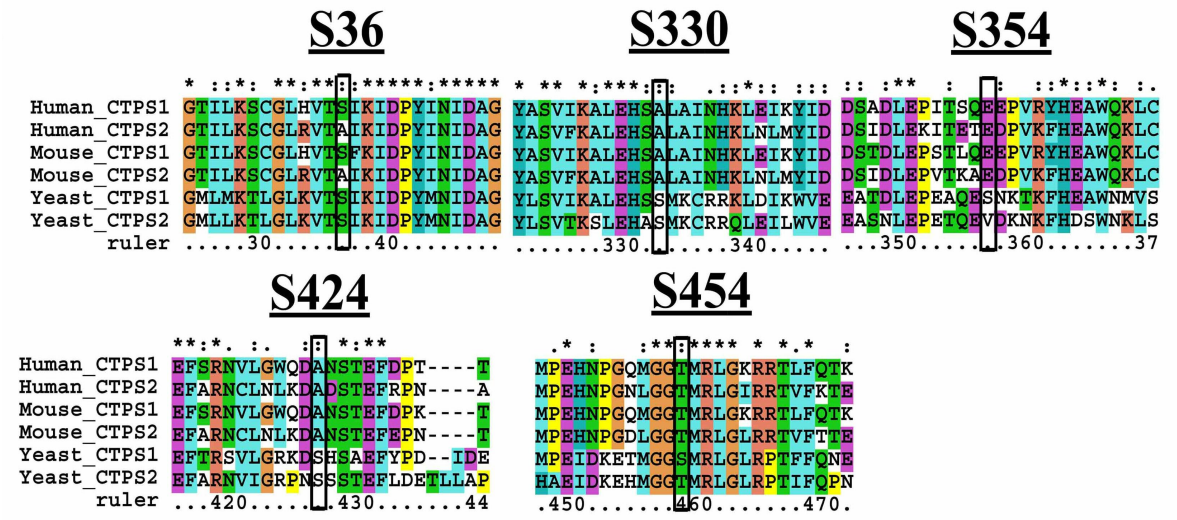


Figure 3.1 Sequence Alignment of Phosphorylation Sites Identified in Yeast

Human (*Homo sapiens)*, mouse *(Mus musculus)*, yeast (*Saccharomyces cerevisiae)* CTPS1 and CTPS2 protein sequences (NCBI accession numbers respectively, NP_001896, Q9NRF8, P70698, P70303, P28274, P38627) were aligned using Clustal X. The indicated yeast phosphorylation sites, using the yeast numbering system, and the surrounding sequences are shown.

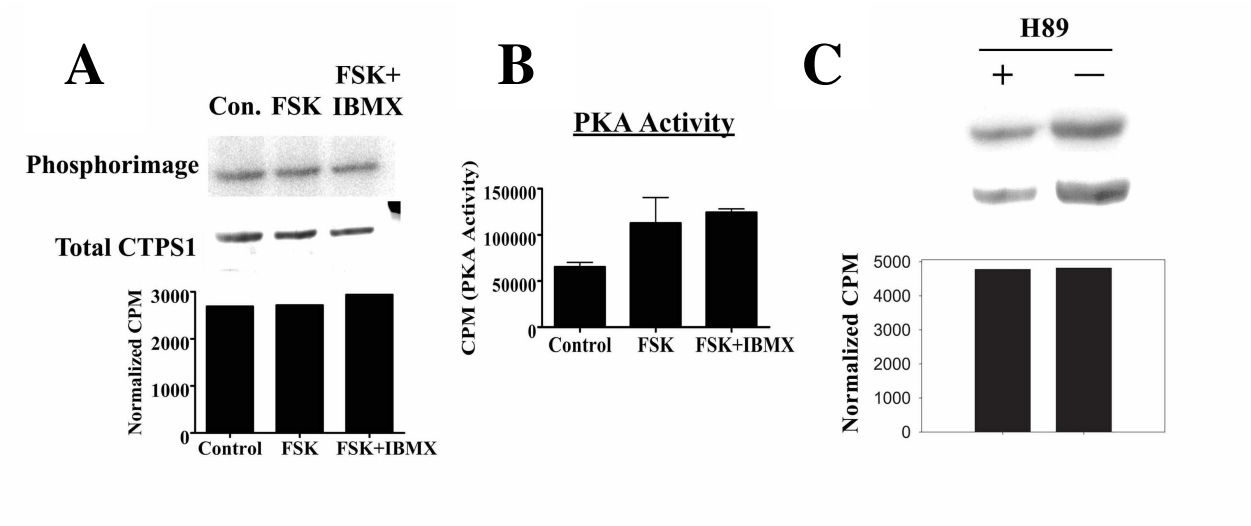


Figure 3.2 Activation or Inhibition of PKA does not Alter CTPS1 Phosphorylation

(A) HEK 293 cells were metabolically labeled with [^{32}P]orthophosphate as described in "Experimental Procedures". Cells were treated with DMSO vehicle, 20 µM Forskolin (FSK) for 15 minutes or 20 µM FSK + 100 µM IBMX for 15 minutes. Immunoprecipitated CTPS1 was resolved by SDS-PAGE, transferred to nitrocellulose and phosphorylated CTPS1 was visualized by phosphorimage analysis. Normalized CPM refers to the CPM from each excised protein normalized to the total protein as determined by densitometry. (B) PKA activity was assessed using KEMPtide as the substrate in an *in vitro* kinase assay as described previously (Kemp et al., 1977). (C) For PKA inhibition, 5 µM H89 was added to HEK 293 cells during the last two hours of [^{32}P]orthophosphate labeling. 20 µM H89 also did not alter CTPS 1 phosphorylation (data not shown).

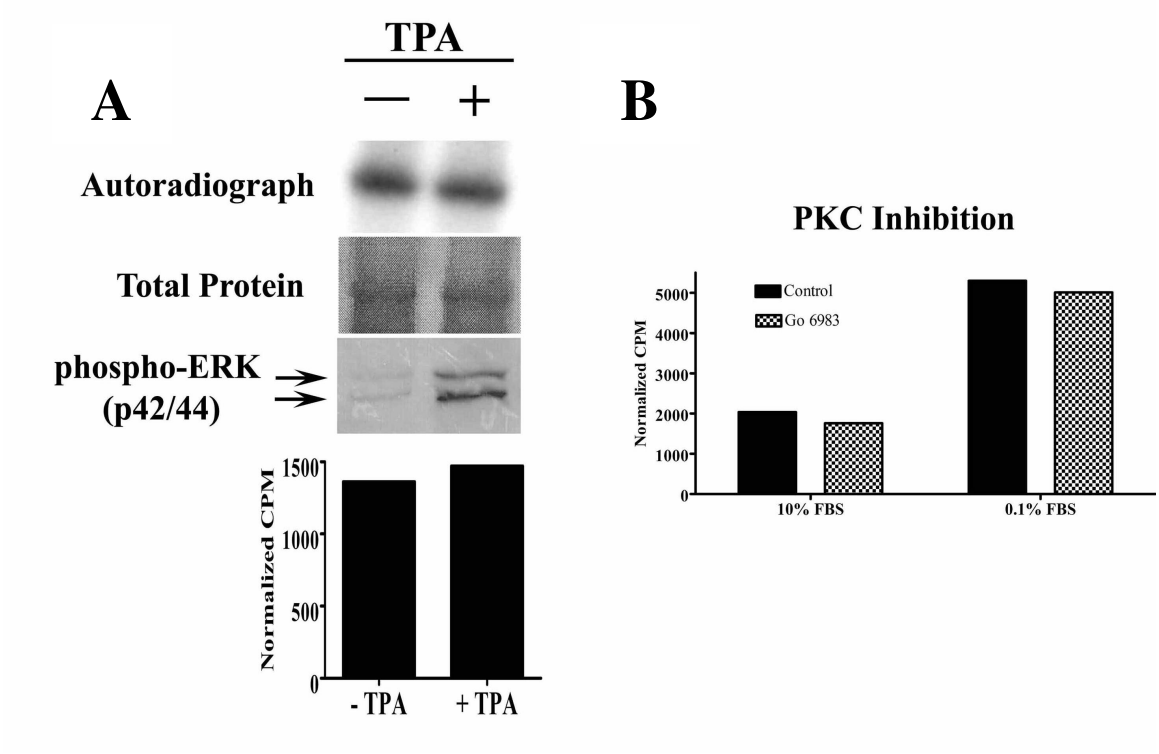


Figure 3.3 Activation or Inhibition of PKC does not alter CTPS1 Phosphorylation

(A) Following [^{32}P]orthophosphate labeling, HEK 293 cells were stimulated with 100 ng/ml PMA for 15 minutes or vehicle control. Activation of PKC was determined by immunoblotting for phosphorylated ERK (Thr202/Tyr204). (B) HEK 293 cells were labeled with [^{32}P]orthophosphate as described in "Experimental Procedures" and treated with 3 μM Go6983 in the presence of 10% FBS or 0.1% FBS. CTPS1 was immunoprecipitated, resolved by SDS-PAGE, transferred to nitrocellulose and phosphorylated CTPS1 was visualized by autoradiography as described in legend for Figure 3.2.

GO 6983 (10 μM)	PKC inh.
GF 109203X (10 μM)	PKC inh.
H89 (10 and 20 μM)	PKA, AMPK, PKG, MSK1, AKT, ROCK-II, S6K1
EGF (100 ng/ml)	EGFR ligand
U0126 (10 μM)	MEK inh.
PD153035 (10 μM)	EGFR inh.
Anisomycin (10 μg/ml)	JNK activator, protein synthesis inh.
SP600125 (10 μM)	JNK inh.
Metformin (2 mM)	AMPK activator
Compound C (10 μM)	AMPK inhibitor
Olomoucine (10 μM)	CDK inhibitor
Roscovitine (10 μM)	CDK inhibitor
2X Thym. HeLa Cell Synchronization	Cell cycle analysis

Taxol (250 nM)	Microtubule stabilizer
Nocodazole (200ng/ml)	Microtubule destabilizer
DMAT (10 μM)	CK II inh.
Indomethacin (2 μM)	COX inh.
Uridine	
NBMPR (10 μM)	ENT 1 inhibitor
CPEC (100 nM)	CTPS inhibitor
H_2O_2 (1 mM)	ROS
UV (50 J/m^2)	DNA damage
Caffeine (3 mM)	DNA damage checkpoint kinases

Table 3.1 Table of Activators, Inhibitors and Stimuli that do not greatly Affect CTPS1 Phosphorylation

Two-dimensional thin layer chromatography (2D TLC) separation of tryptic peptides from endogenous CTPS1 revealed five major phosphopeptide spots and consistent with the effects of low-serum on CTPS phosphorylation, all five of these spots increased after serum starvation (Figure 3.4 A).

Since 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 (AMPK) and glycogen synthase kinase 3 (GSK3) (Munday et al., 1991),(Welsh et al., 1994). Incubation of HEK 293 cells with the AMPK 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 (Ser21, Ser9 respectively) was decreased in 0.1% FBS, indicating that GSK3 was more active when HEK 293 cells were maintained overnight in low serum (Figure 3.4 B). 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 (DMSO) overnight in the presence of 10% FBS 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 (Figure 3.4 C). Furthermore, two-dimensional phosphopeptide mapping showed that indirubin decreased the radioactivity of the five phosphopeptide spots equally (Figure 3.4 C). 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) (Figure 3.4 D). GSK3β siRNA almost completely eliminated GSK3β expression, but did not effect GSK3α

expression (Figure 3.4 D). Analysis of CTPS1 phosphorylation in these cells demonstrated that low-serum induced CTPS1 phosphorylation was inhibited in cells transfected with GSK3β siRNA compared to cells transfected with non-targeting siRNA (Figure 3.4 D).

Although much of the low-serum induced CTPS1 phosphorylation was inhibited by GSK3β siRNA, the low-serum induced CTPS1 radioactivity was not reduced as significantly as that observed after indirubin treatment (Figure 3.4 C and D), suggesting that GSK3α may also contribute to the phosphorylation of CTPS1.

3.3.2.2 Identification of S574 and S575 as hCTPS1 phosphorylation sites in intact cells

To identify the amino acids phosphorylated in endogenous CTPS1, HEK 293 cells were labeled with [^{32}P]orthophosphate and CTPS1 was isolated by immunoprecipitation. Tryptic peptides from endogenous CTPS1 were separated by 2D TLC and the five phosphopeptide spots routinely observed were scraped, the peptides eluted and analyzed by nano-electrospray ionization tandem mass spectrometry (nano-ESI-MS/MS). Spot 3 was identified as a C-terminal tryptic peptide (SGSSS*PDSEITELK, "*" denotes phosphorylation) containing a phosphorylation on serine 575 (S575) (Figure 3.5 A). Spots 1 and 5 were identified as the same peptide isolated but the MS/MS data was consistent with a doubly phosphorylated peptide (SGSS*S*PDSEITELK), S574 and S575 (Figure 3.5 A). 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 (Figure 3.5 B) substantiating the identification of this spot as a tryptic peptide containing phosphorylated S574 and S575.

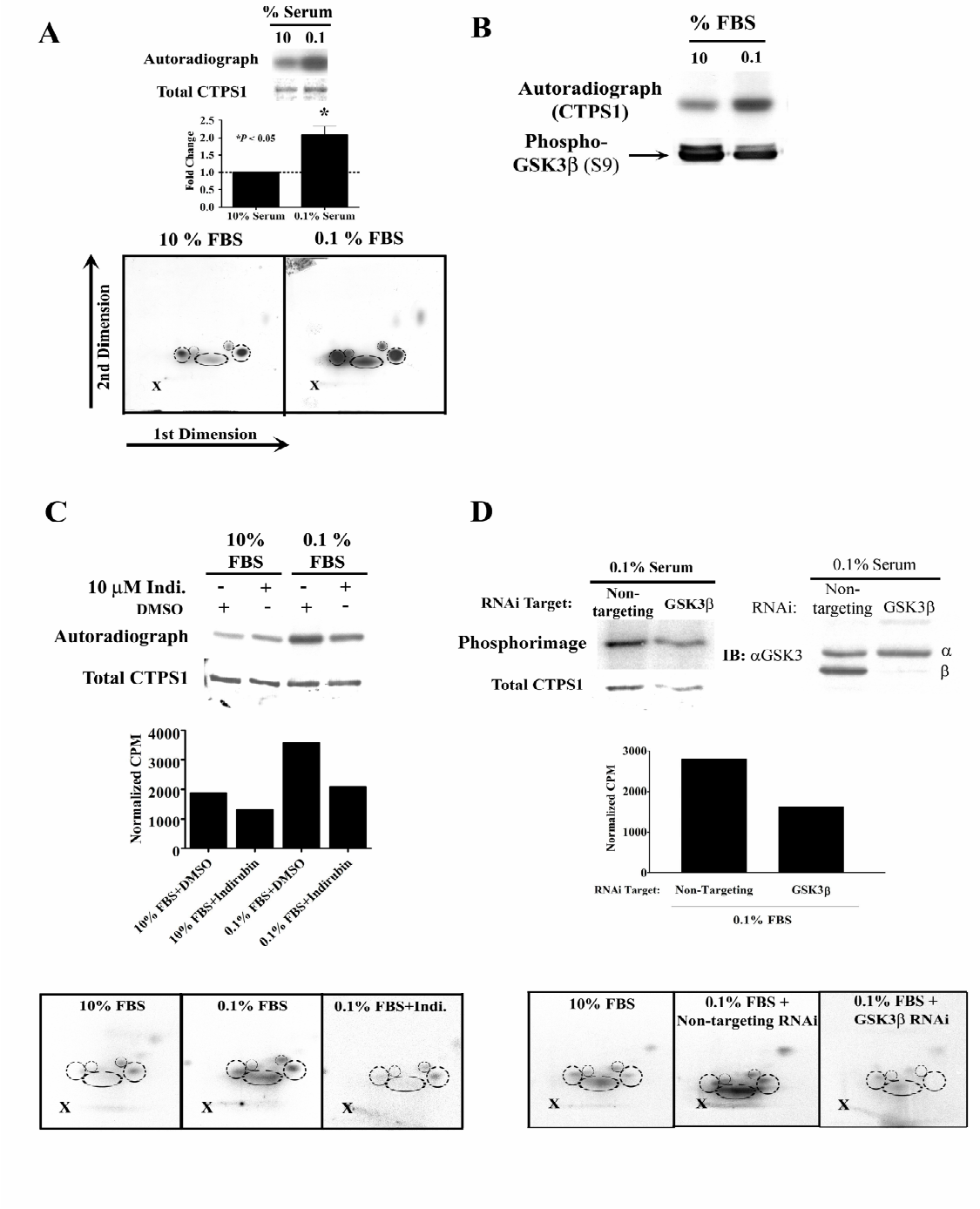


Figure 3.4 Involvement of GSK3β in phosphorylation of endogenous human CTPS1

(A) HEK 293 cells were maintained in 0.1% or 10% FBS overnight and then labeled with [^{32}P]orthophosphate containing 0.1% or 10% FBS. Lysates were prepared and CTPS1 immunoprecipitated as described in "Experimental Procedures". Immunoprecipitated CTPS1 was separated by SDS-PAGE, transferred to nitrocellulose and exposed to X-ray film. Autoradiograph and two-dimensional TLC separation of tryptic peptides are representative of

four experiments. Total CTPS1 was visualized by MemCode staining the nitrocellulose membrane. Fold-change bar graph uses data from four experiments (n=4) (B) To determine the status of GSK3 activation, serine 9 phosphorylation of GSK3β was assessed by immunoblotting lysates from [^{32}P]orthophosphate labeled HEK 293 cells maintained overnight in 10% or 0.1% FBS. Lysates were prepared as described in "Experimental Procedures". (C) HEK 293 cells maintained overnight in 10% or 0.1% FBS in the presence of vehicle (DMSO) or 10 μM indirubin-3-monoxime were [^{32}P]orthophosphate labeled as described in "Experimental Procedures". Cells were lysed, CTPS1 immunoprecipitated and visualized as in Fig. 1A. Radioactivity associated with CTPS1 was determined by excising the corresponding CTPS1 band, Cerenkov counting radioactivity and adjusting the counts per minute (CPM) for protein amount as described in "Experimental Procedures". CTPS1 bands were trypsin digested and two-dimensional thin layer chromatography was performed as described in "Experimental Procedures". (D) HEK 293 cells were transfected with the indicated SmartPool RNA duplexes from Dharmacon as described in "Experimental Procedures". Two days following transfection of RNA duplexes, media was switched to 0.1% FBS overnight and cells were labeled the following day with [^{32}P]orthophosphate. CTPS1 was immunoprecipitated, separated and visualized as described in Fig. 1C. Two-dimensional thin layer chromatography was also performed as described in Fig. 1C. Reduction in GSK3β protein expression was assessed by immunoblotting with an antibody that recognizes total GSK3α and GSK3β.

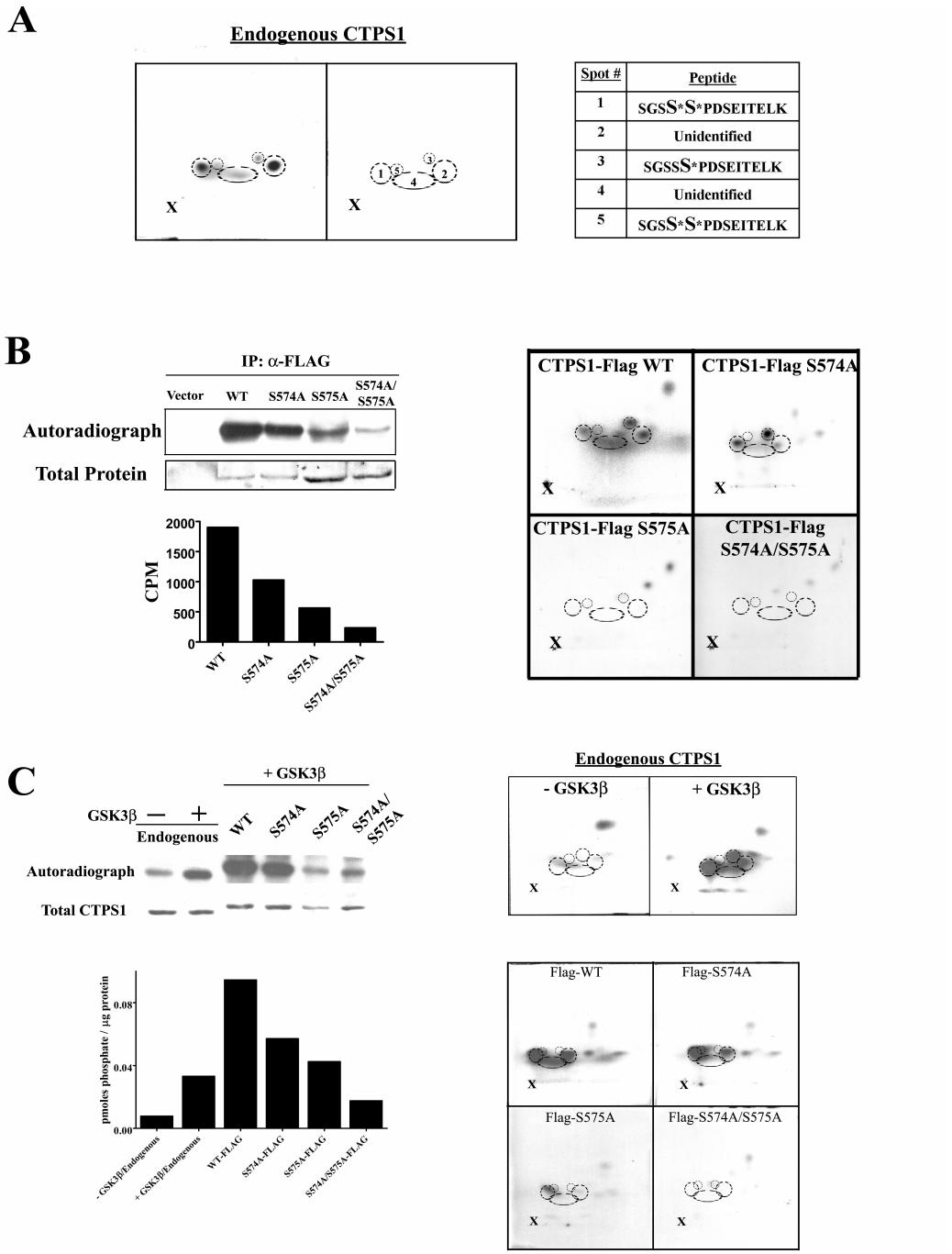


Figure 3.5 Identification of S574 and S575 as Phosphorylation Sites in Human CTPS1 and Involvement of S574 and S575 in GSK3β Phosphorylation of CTPS1.

(A) HEK 293 cells were labeled with [^{32}P]orthophosphate as described in "Experimental Procedures" and CTPS1 was immunoprecipitated and visualized as described in Fig. 1C. Trypsinized endogenous CTPS1 peptides were subjected to two-dimensional thin layer chromatography as described previously. Radioactive spots observed on

autoradiograph from TLC plates were scraped. Phosphopeptides eluted from TLC plate backing and subjected to nano-ESI MS/MS for identification as described in "Experimental Procedures". (B) Wild type (WT) FLAG-CTPS1 and indicated FLAG-CTPS1 phosphorylation site mutants were immunoprecipitated using anti-FLAG resin from transfected HEK 293 cells labeled with [^{32}P]orthophosphate and separated by SDS PAGE. Following transfer to nitrocellulose, bands corresponding to FLAG-CTPS1 were excised and radioactivity was counted using Cerenkov counting. Peptides eluted from trypsinized FLAG-CTPS1 WT and phosphorylation site mutants were separated using two-dimensional thin layer chromatography as described in "Experimental Procedures". (C) WT FLAG-CTPS1 or FLAG-CTPS1 phosphorylation site mutants expressed in HEK 293 cells were immunoprecipitated using anti-FLAG resin as described in "Experimental Procedures". Endogenous CTPS1 was immunoprecipitated as detailed in "Experimental Procedures". Endogenous CTPS1 and immunoprecipitated FLAG-CTPS1 was used as substrates in an *in vitro* GSK3β kinase reaction as detailed in "Experimental Procedures". Following the *in vitro* GSK3β kinase reaction, endogenous CTPS1 and indicated FLAG-CTPS1 proteins were separated by SDS PAGE, transferred to nitrocellulose and visualized by autoradiography. Radioactivity associated with CTPS1 was determined as in Fig. 1C. Endogenous CTPS1 and FLAG-CTPS1 proteins were trypsin digested and tryptic peptides were subjected to separation by two-dimensional thin layer chromatography as described in "Experimental Procedures".

The S575A mutant showed decreased intensity of all the spots, except for spot 3 which was barely detectable, suggesting that S575 was a major phosphorylation site and/or that it was required to "prime" for additional phosphorylations (Figure 3.5 B). Mutation of S574, S575 or both S574/S575 to alanine (S574A, S575A S574A/S575A) significantly decreased the amount of radioactivity associated with CTPS1 compared to wild type (WT) CTPS1 (Figure 3.5 B). Mutation of both S574 and S575 to alanines almost completely eliminated the radioactivity associated with CTPS1 indicating that S574 and S575 are two major phosphorylation sites in CTPS1 (Figure 3.5 B).

3.3.2.3 Requirement of S574 and S575 for phosphorylation of hCTPS1 by GSK3β directly

To determine whether GSK3β could phosphorylate S574 and/or S575 directly, CTPS1 or the indicated phosphorylation site mutants were immunoprecipitated and used as substrates in an *in vitro* kinase reaction with GSK3β (Figure 3.5 C). 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. This data showed the ability of GSK3β to phosphorylate CTPS1 and indicated that S574 and, to a greater extent, S575 were necessary for the direct phosphorylation of CTPS1 by GSK3β (Figure 3.5 C). Additionally, these phosphopeptides were separated by 2D TLC to generate phosphopeptide maps and the pattern of spots compared to the pattern observed from CTPS1 phosphorylated in intact cells (compare phosphopeptide maps from Figure 3.4 A to phosphopeptides maps from Figure 3.5 C). This data strongly suggested that GSK3β was directly phosphorylating CTPS1 on sites similar to those observed *in vivo*.

3.3.2.4 Identification of S571 as a hCTPS1 phosphorylation site in intact cells

GSK3 is a hierarchal 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), X is any amino acid) (Fiol et al., 1988). The large decrease in phosphorylation observed with the mutation S575A, both in intact cells and *in vitro* with GSK3β, implicates S575 as a critical residue involved in GSK3 phosphorylation of CTPS1. However whether S575 is serving as the actual GSK3 phosphorylation site or the priming site for GSK3 phosphorylation of S571 could not be determined from the results in Figure 3.5. To determine whether the negative charge of the glutamate at residue 579 (E579) was serving as a phosphomimetic residue and priming for GSK3 phosphorylation of S575, 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 E579 does not prime for GSK3 phosphorylation of S575 in intact cells (Figure 3.6). Another possibility was that phosphorylation of S575 could prime for GSK3 phosphorylation of S571. Interestingly, the CTPS1 clone we received from the IMAGE consortium contained a mutation of S571 to an isoleucine (S571I). To address whether S571 was a phosphorylation site in CTPS1 in intact cells, the S571I mutation was expressed in HEK 293 cells labeled with [^{32}P]orthophosphate. Following isolation by anti-FLAG immunoprecipitation, the CTPS1 S571I mutant had substantially less radioactivity associated with it compared to WT CTPS1-FLAG, indicating that S571 was required for CTPS1 phosphorylation (Figure 3.7 A). The combined mutation of S575 and S574/S575 to alanine with S571I decreased the radioactivity associated with CTPS1 suggesting that S575 was another major site labeled under these conditions (Figure 3.7 A).

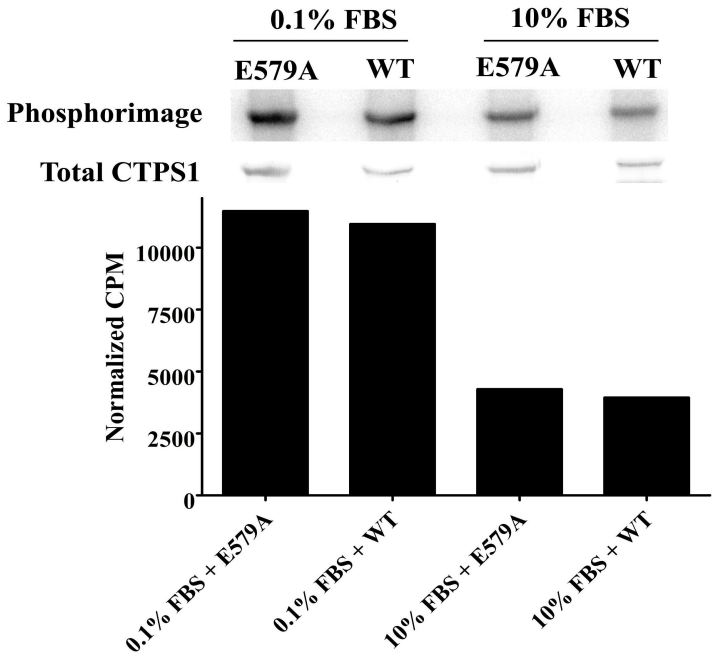


Figure 3.6 E579A Mutation of CTPS1 does not Alter CTPS1 Phosphorylation

HEK 293 cells were transfected with the indicated DNA, labeled with [^{32}P]orthophosphate and Flag-tagged CTPS1 was isolated as described earlier in the legend to Figure 3.5. Radioactivity (CPM) associated with each protein was normalized to total protein observed by densitometry.

2D TLC revealed that when S571 was mutated to an isoleucine, the majority of radioactive spots were diminished, with the exception of one prominent spot (Figure 3.7 A). This phosphopeptide spot did not disappear when S574 was mutated to alanine in combination with S571I, but was absent from the S571I/S575A double mutant, indicating that the phosphopeptide that gives rise to this spot is dependent on S575 being phosphorylated (Figure 3.7 A). Because serine 571 is mutated to a more hydrophobic residue, (isoleucine), phosphorylated peptides containing this isoleucine would migrate further 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 S571 was mutated to an isoleucine most likely corresponds to spots 2 or 3. Consistent with this, spot 3 was identified earlier as the same tryptic peptide containing unphosphorylated S571 and singly phosphorylated S575 (See Figure 3.5 A).

3.3.2.5 Involvement of S571 in the phosphorylation of hCTPS1 by GSK3β directly

To determine whether S571 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 S571 was indeed a phosphorylation site for GSK3β (Figure 3.7 B).

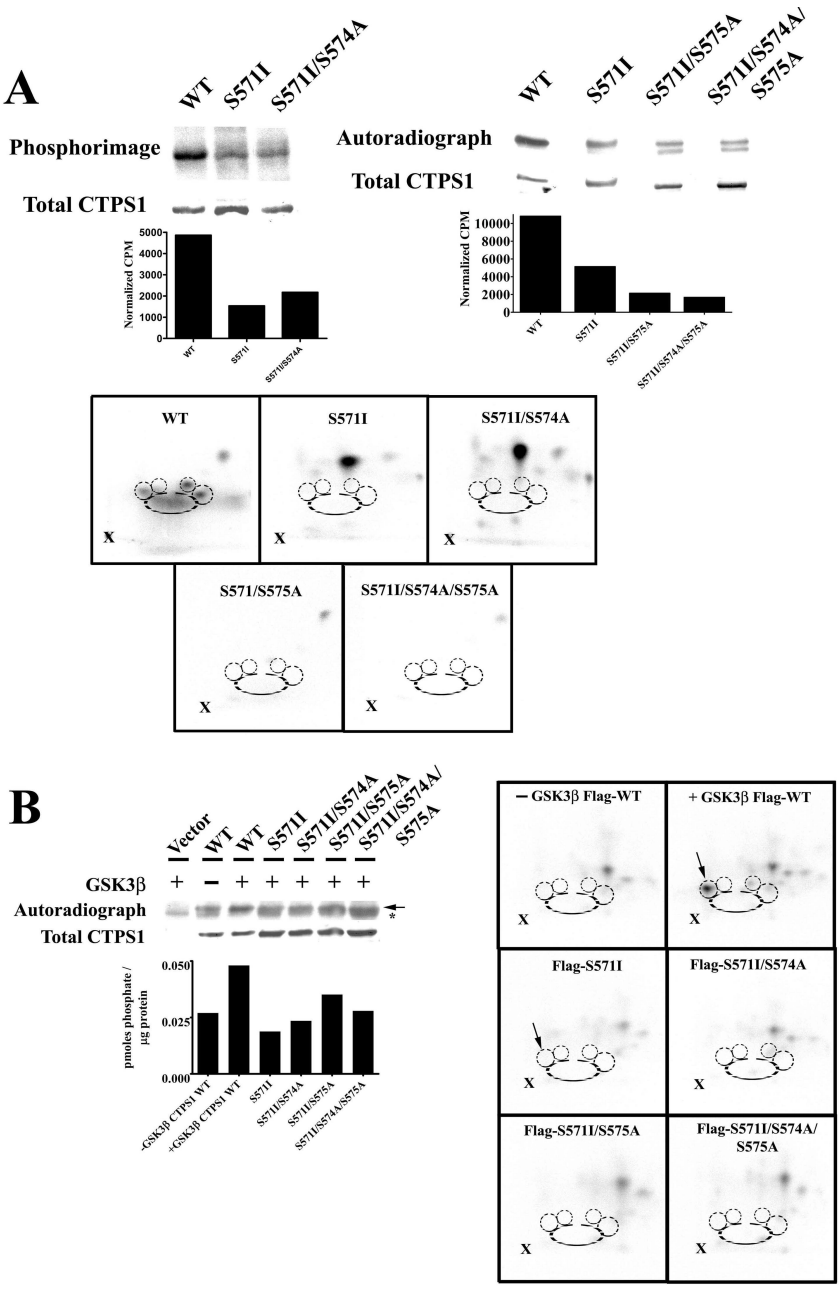


Figure 3.7 Identification of S571 as a Phosphorylation Site in Human CTPS1 and Involvement of S571 in GSK3β Phosphorylation of CTPS1

(A) As in Fig. 1B, HEK 293 cells were transfected with the indicated DNA. Following [^{32}P]orthophosphate labeling of cells, radiolabeled CTPS1 protein was immunoprecipitated, separated by SDS PAGE, transferred to nitrocellulose and visualized either by autoradiography or phosphorimage analysis. Tryptic peptides were separated by two-dimensional thin layer chromatography as described in "Experimental Procedures". (B) As in Fig. 2C, WT FLAG-CTPS1 and indicated FLAG tagged CTPS1 phosphorylation site

mutants were used as substrates in an *in vitro* GSK3β kinase reaction. *In vitro* GSK3β phosphorylation of WT and CTPS1 phosphorylation site mutants were analyzed by autoradiography, Cerenkov counting and two-dimensional thin layer chromatography as described in Fig. 2C. Unlike in Fig. 2C only a quarter of the isolated peptides were loaded onto the TLC plate to avoid overloading the plate with radioactivity. "*" indicates a non-specific immunoprecipitated protein observed in the autoradiograph migrating just below FLAG-CTPS1. Arrow indicates FLAG-CTPS1.

Analysis of the S571I/S574A phosphorylation site mutant did not show altered incorporation of phosphate compared to S571I alone, but analysis of S571I/S575A slightly elevated the amount of phosphate incorporated into CTPS1 compared to S571I, suggesting that without the ability to phosphorylate S571 or S575 *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 decreased in the amount of phosphate incorporated into CTPS1 compared to S571I/S575A suggesting that S574 may serve as a minor secondary site for GSK3β phosphorylation *in vitro* (Figure 3.7 B). Separation of phosphorylated peptides from the *in vitro* GSK3β kinase reaction by 2D TLC demonstrated that spots 1 and 5 were the most intense (See arrow in Figure 3.7 B). Although, we previously found the doubly phosphorylated (S574 and S575) 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 S571 was phosphorylated. As expected, mutation of S571 to isoleucine (S571I) abolished the appearance of this spot (see arrow in Figure 3.7 B), demonstrating that this spot contains phosphorylated S571 and that S571 is the major site in CTPS1 for GSK3β. Since 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. Since GSK3β requires priming phosphorylation, this spot (arrow in Figure 3.7 B) contains a peptide with at least two phosphorylated residues, S571 and S575 (Figure 3.7 B). Further mutation of S574,

S575 and both S574/S575 to alanine combined with S571I did not change the results observed with S571I alone, again demonstrating that S571 was the major target of GSK3β *in vitro*.

3.3.2.6 Effect of GSK3 and phosphorylation on CTPS1 activity

Most GSK3 substrates are negatively regulated by GSK3 phosphorylation (Beals et al., 1997; Benjamin et al., 1994; Boyle et al., 1991; Dent et al., 1989; Diehl et al., 1998; Eldar-Finkelman and Krebs, 1997; Fiol et al., 1988; Hughes et al., 1992; Morfini et al., 2002; Pulverer et al., 1994; Yost et al., 1996). To examine if CTPS1 activity 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 to cells incubated with 10% FBS (Figure 3.8 A), 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 (Figure 3.8 A), consistent with GSK3 phosphorylation inhibiting the activity of CTPS1.

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 minutes with calf intestinal phosphatase (CIP) and CTPS1 activity was measured (Figure 3.8 B). CTPS1 was significantly dephosphorylated after 15 minutes of incubation with CIP as seen by the phosphorimage of total isolated CTPS1 and in the 2D phosphopeptide map (Figure 3.8 B).

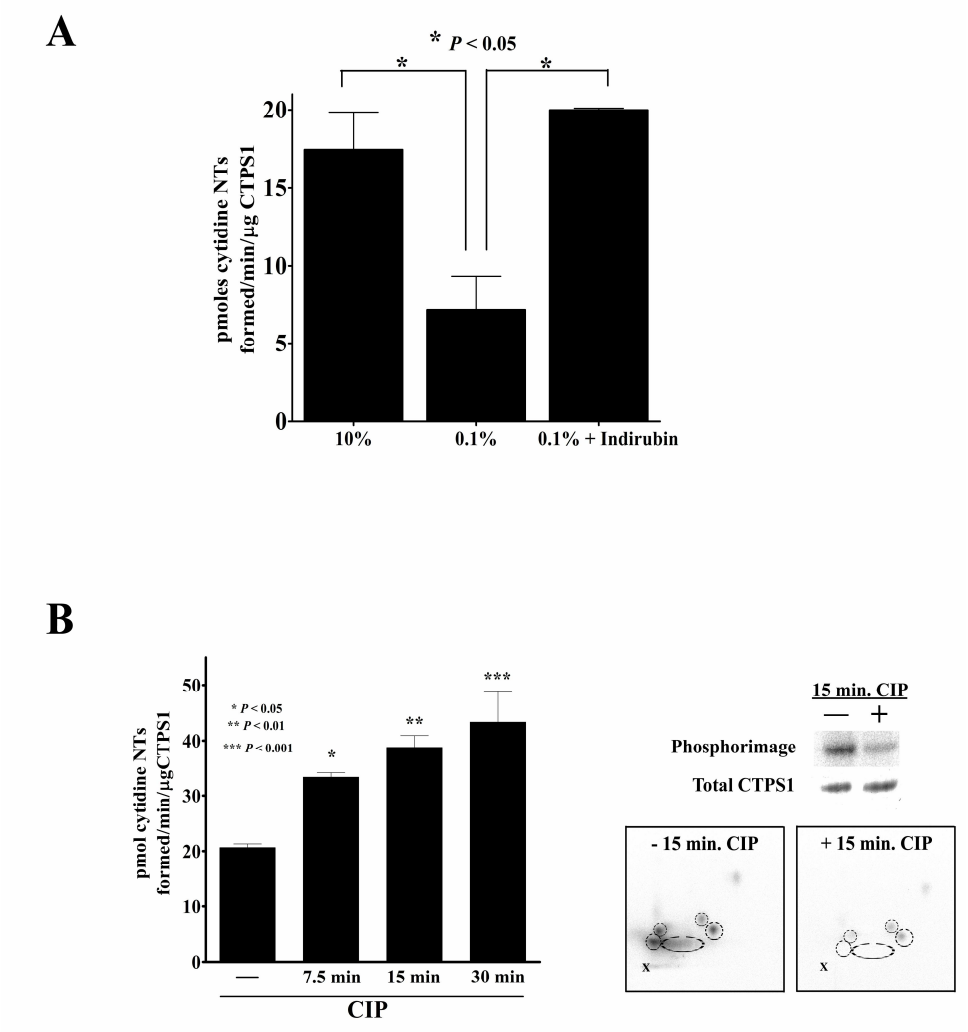


Figure 3.8 Effect of Serum, Indirubin and Phosphatase Treatment on CTPS1 Activity.

(A) Endogenous CTPS1 was immunoprecipitated from HEK 293 cells maintained overnight in 10% FBS, 0.1% FBS or 0.1% FBS with 10 µM indirubin (indirubin-3'-monoxime). CTPS1 activity was determined as described in "Experimental Procedures". Statistical analysis was done using one-way ANOVA (95% confidence interval) and a Tukey posthoc test. $P < 0.05$ was considered significant and designated by "*". (B) Endogenous CTPS1 was immunoprecipitated from HEK 293 cells maintained overnight in 0.1% FBS. Immunoprecipitated endogenous CTPS1 was treated for the indicated times with 15 Units of calf intestinal alkaline phosphatase (CIP) or equivalent volume of CIP suspension buffer (50% glycerol) as a control for 30 minutes as detailed in "Experimental Procedures". Following CIP treatment, CTPS1 was washed with 1X CTPS reaction buffer and CTPS activity was assayed as described in "Experimental Procedures". Statistical analysis was done using one-way ANOVA (95 % confidence interval) and a Tukey posthoc test. $P < 0.05$, $P < 0.01$ and $P < 0.001$ were considered significant and indicated by "*", "**" and "***", respectively. Endogenous CTPS1 from [^{32}P]orthophosphate labeled HEK 293 cells maintained in 0.1% FBS overnight was immunoprecipitated and treated with CIP or

equivalent amount of 50% glycerol as a control for 15 minutes. Radioactivity associated with CIP and control treated endogenous CTPS1 was visualized by phosphorimage. CIP and control treated CTPS1 was also analyzed by two-dimensional thin layer chromatography as previously described in Fig. 1C and in detail in “Experimental Procedures”.

CTPS1 activity increased with the time of incubation with CIP demonstrating that CTPS1 was more active following dephosphorylation of the enzyme (Figure 3.8 B).

3.3.3 Further Regulation of Human CTPS1 by Phosphorylation

3.3.3.1 hCTPS1 is not Phosphorylated by JNK, ERK or During the Cell Cycle

Our results demonstrate that GSK3 phosphorylation of hCTPS1 required a priming phosphorylation on S575, which is N-terminal to a proline, suggesting a proline-directed kinase phosphorylated this site. Since many of the processes that use CTP (e.g. DNA/RNA synthesis, phospholipid synthesis, etc.) are involved in cell proliferation we tested whether the proline-directed kinases of the MAPK family or the CDK family were involved in hCTPS1 phosphorylation. To determine whether the MAPK, ERK, was involved in CTPS1 phosphorylation, serum-starved HEK 293 cells were labeled with [^{32}P]orthophosphate and stimulated with 100 ng/ml epidermal growth factor (EGF) for 10 minutes or pre-treated for 5 minutes with the MEK inhibitor, U0126 (10 μM), prior to EGF stimulation. Despite activation of ERK by EGF or suppression of ERK activation by pre-treatment with U0126, phosphorylation of endogenous hCTPS1 was not changed (Figure 3.9). This result was consistent with a large-scale analysis of phosphorylation sites in HeLa cells stimulated with EGF where none of the phosphorylation sites identified in hCTPS1 changed with EGF treatment (Olsen et al., 2006). To investigate whether the MAPK c-Jun N-terminal kinase, JNK, was involved in hCTPS1 phosphorylation, HEK 293 cells labeled with [^{32}P]orthophosphate were stimulated with anisomycin, a JNK activator, and the phosphorylation of hCTPS1 was assayed as described earlier.

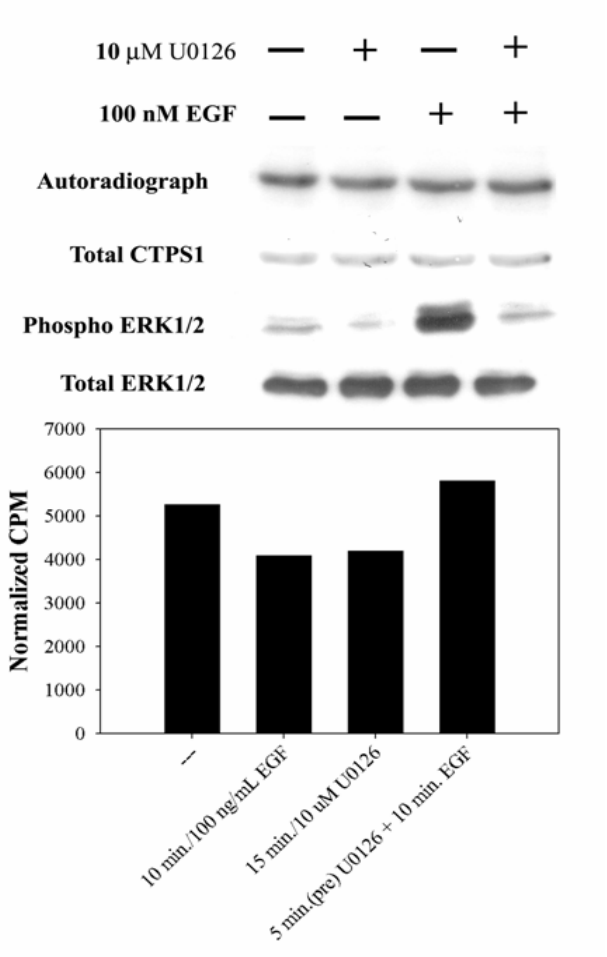


Figure 3.9 ERK is not Involved in hCTPS1 Phosphorylation

HEK 293 cells were maintained in 0.1% FBS overnight and then stimulated with 100 ng/ml EGF in the presence or absence of the MEK inhibitor U0126 (10 μM). Phosphorylation of CTPS1 was analyzed as described in "Experimental Procedures". Activation of ERK was assessed by immunoblotting for phosphorylation of Thr202/Tyr204 on ERK. Normalized CPM described in legend for Figure 3.2.

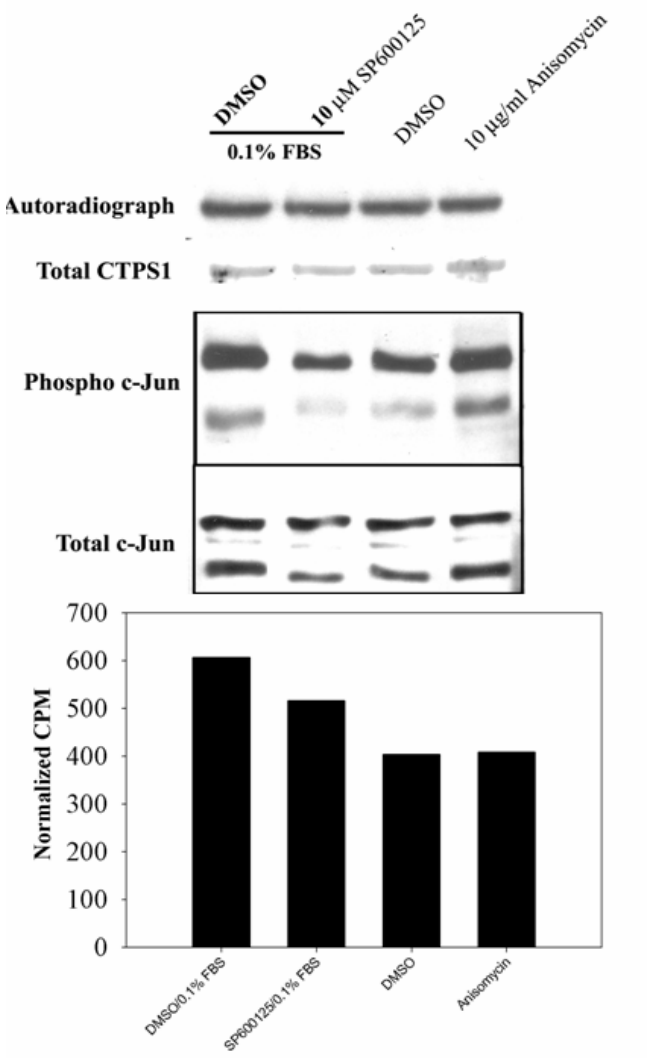


Figure 3.10 JNK is not Involved in hCTPS1 Phosphorylation

HEK 293 cells were labeled with [^{32}P]orthophosphate and then stimulated with 10 µg/ml anisomycin or DMSO for 1 hour. For SP600125 treatment, HEK 293 cells were maintained in 0.1% FBS overnight in the presence of 10 µM SP600125 or DMSO and labeled with [^{32}P]orthophosphate. Phosphorylation of CTPS1 was assessed as described in "Experimental Procedures". JNK activity was assessed by immunoblotting for the phosphorylation of c-Jun (Ser73), a JNK substrate.

Despite activation of JNK as shown by phosphorylation of c-Jun, no change in the phosphorylation of hCTPS1 was observed (Figure 3.10).

To further determine if JNK was involved in the low-serum induced phosphorylation, cells were incubated overnight in the absence or in the presence of the JNK inhibitor SP600125 (10 μM). Incubation with the JNK inhibitor did not alter hCTPS1 phosphorylation indicating that JNK was not involved in low-serum induced phosphorylation and unlikely to be necessary for priming phosphorylation on S575 (Figure 3.10).

The involvement of cell cycle-dependent kinases, CDKs, was investigated by synchronizing HeLa cells using double thymidine block and [^{32}P]orthophosphate labeling cells. Although HeLa cells were synchronized as observed by cyclin A protein levels, CTPS1 phosphorylation did not change throughout the cell cycle (Figure 3.11). Furthermore, treatment of [^{32}P]orthophosphate labeled HEK293 cells with the CDK inhibitors roscovitine and olomoucine (data not shown) did not alter hCTPS1 phosphorylation indicating that CDKs 1, 2 do not seem to be involved in the phosphorylation of hCTPS1.

3.3.3.2 Involvement of CK I in hCTPS1 Phosphorylation

All of the phosphorylation sites of hCTPS1 in the Phosida database are confined to the C-terminal 35 amino acids of the protein and contain many consensus CK I phosphorylation sites (Flotow et al., 1990; Olsen et al., 2006). To test whether CK I is involved in the phosphorylation of hCTPS1, HEK 293 cells were labeled with [^{32}P]orthophosphate and either maintained in 10% or 0.1% serum in the presence or absence of the CK I inhibitor D4476.

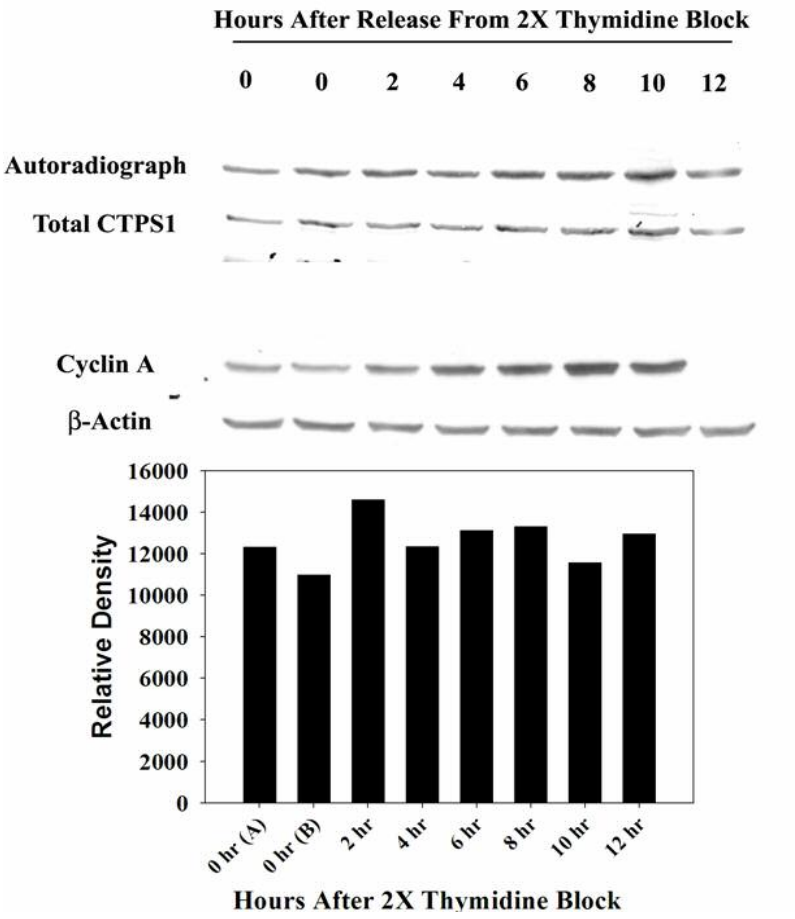


Figure 3.11 hCTPS1 Phosphorylation is not Altered During the Cell Cycle

HeLa cells were synchronized by double thymidine block and 2 hours prior to harvesting cells, cells were labeled with [^{32}P]orthophosphate. Phosphorylation of CTPS1 was determined by determining the relative density of the autoradiograph to the corresponding amount of total CTPS1. Synchronization of the cell cycle was ascertained by immunoblotting for cyclin A. Total amount of protein was determined by immunoblotting for β-actin.

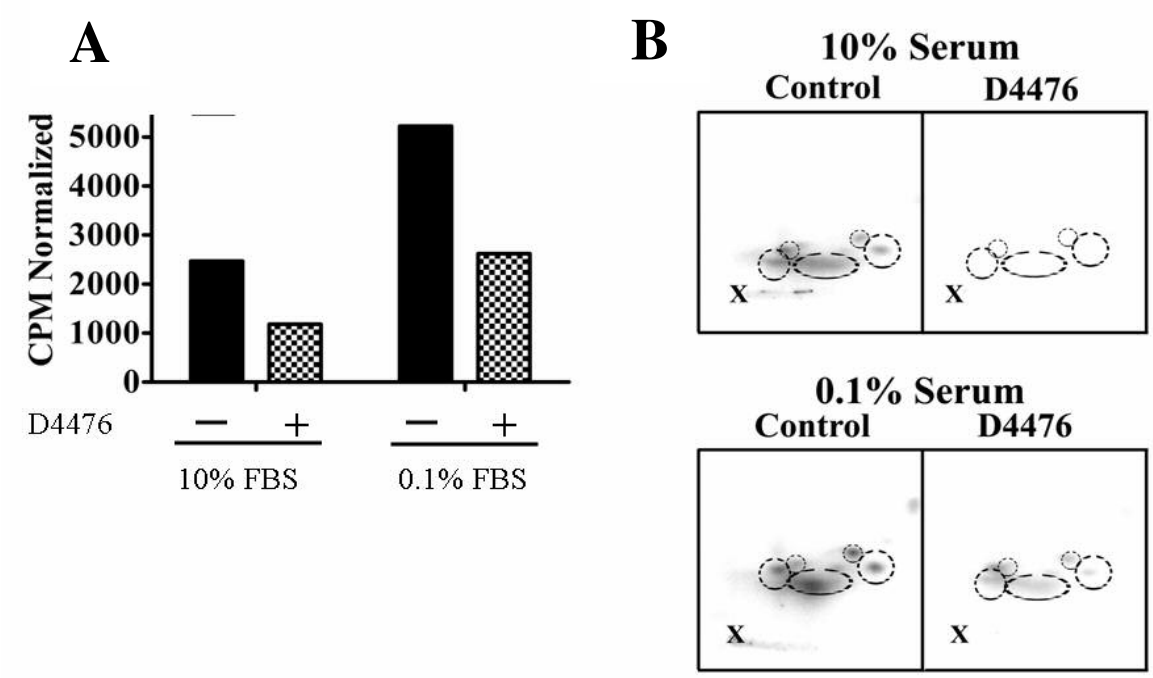


Figure 3.12 Inhibition of CK I Reduces Phosphorylation of hCTPS1

(A) HEK 293 cells were maintained in 10% or 0.1% FBS overnight in the presence or absence of the CK I inhibitor D4476 (50 μM). Following [^{32}P]orthophosphate labeling of HEK 293 cells, CTPS1 was isolated as described in "Experimental Procedures". Shown is the radioactivity of CTPS1 normalized to total amount of CTPS1. (B) Also shown are the separation of the tryptic peptides by 2D TLC.

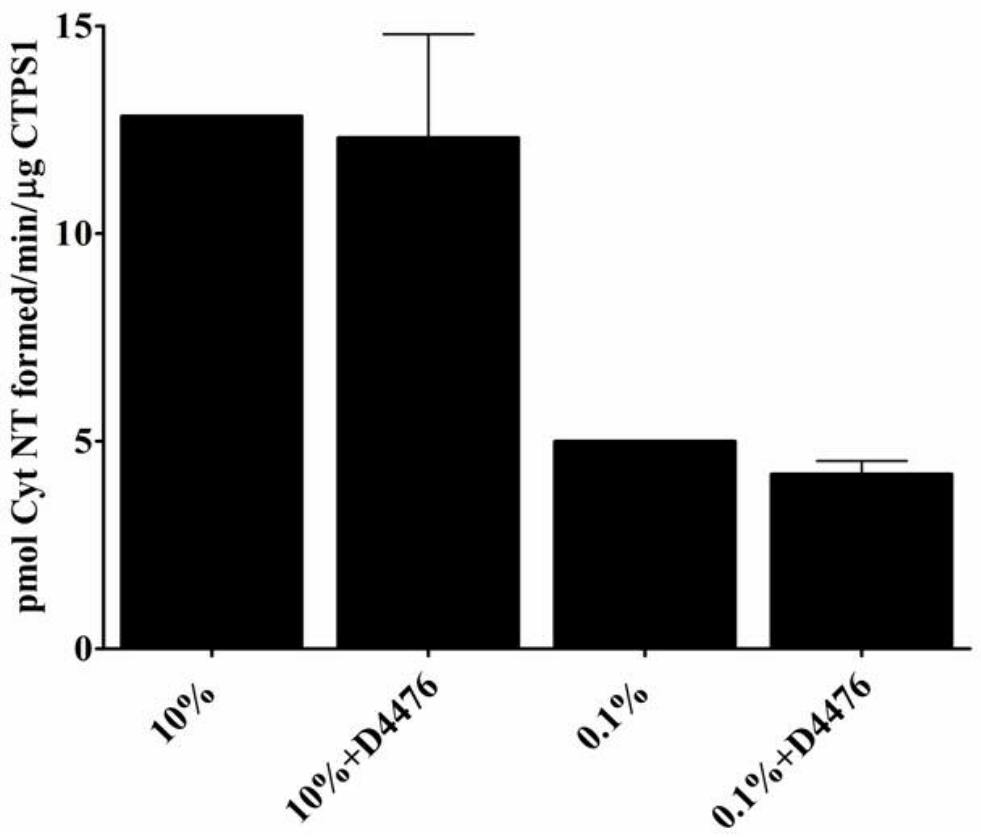


Figure 3.13 CK I Inhibition does not Affect CTPS1 Activity

HEK 293 cells were maintained in 10% or 0.1% FBS overnight in the presence or absence of the CK I inhibitor, D4476 (50 µM). CTPS1 activity was determined as described in "Experimental Procedures".

The CK I inhibitor D4476 reduced phosphorylation in both 10% and 0.1% maintained HEK 293 cells consistent with the idea that CK I is constitutively active and is involved in phosphorylating CTPS1 under 10% and 0.1% serum conditions (Figure 3.12). Separation of tryptic peptides control and D4476 treated HEK 293 cells showed a decrease in intensity of multiple spots suggesting that CK I phosphorylates hCTPS1 on multiple residues (Figure 3.12). CK I phosphorylation of hCTPS1 on multiple residues is not surprising given the fact that CK I is a hierarchal kinase that requires a priming phosphorylation or an acidic residue at the residue n-2 from the CK I phosphorylation site ((priming-phospho-S/T)XX(S/T-CK I – phospho)) (Flotow et al., 1990) and that many sites in the C-terminus of hCTPS1 resemble consensus CK I phosphorylation sites (See Figure 3.14).

3.3.3.3 Inhibition of CK I does not Affect CTPS1 Activity Under the Conditions Tested

To investigate whether CK I was involved in hCTPS1 activity, HEK 293 cells were maintained overnight in 10% or 0.1% FBS in the presence or absence of the CK I inhibitor D4476. CK I inhibition did not alter hCTPS1 enzyme activity under the conditions tested (Figure 3.13) suggesting that if CK I is involved in hCTPS1 phosphorylation it may regulate a different CTPS function (e.g. localization) or may regulate hCTPS1 under conditions different from the one it was tested in.

3.4 Conclusions

Although the phosphorylation of both yeast and human CTPS1 expressed in yeast has been investigated, this is the first investigation of the phosphorylation of human CTPS1 in human cells. Unlike yeast CTPS1, endogenous human CTPS1 was not phosphorylated by

PKA or PKC under the conditions examined in this study. This is consistent with the fact that many of the PKA and PKC sites found in yeast CTPS1, are not conserved in human CTPS1 (Figure 3.1). Instead our results point to the novel and unexpected phosphorylation of the carboxy terminus of human CTPS1. None of the putative sites in the carboxy terminus of human CTPS1 are consensus PKA or PKC sites, consistent with our results. Sequence alignment of the carboxy termini from various organisms shows how conserved many of the phosphorylation sites are among mammals, whereas in other eukaryotes they diverge (e.g. fly, worm, yeast) (Figure 3.14).

The observation that the sequence conservation varies between mammals and *D. melanogastor* and *C. elegans* does not preclude that *D. melanogastor* and *C. elegans* are regulated by phosphorylation on their carboxy terminus, but does suggest that the regulation of CTPS1 by phosphorylation may be different in these other organisms. The difference between the carboxy terminus from yeast and human CTPS1 suggests that human CTPS1, and perhaps nucleotide metabolism, is regulated differently in humans than in yeast. S571 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 of this isoform.

In human and mouse CTPS, S575 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 of S575 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 S575 to alanine inhibited much of the radioactivity associated with CTPS1 and S575 was investigated as a direct GSK3 phosphorylation site by mutating the potential priming site, E579, to alanine (E579A). Since the E579A mutant failed to alter CTPS1 phosphorylation, S575 was investigated as a priming site for phosphorylation of S571. From these studies it was concluded that S571 was the major site for GSK3 phosphorylation *in vitro*. Interestingly, the CTPS1 clone we received from the IMAGE consortium, derived from a retinitis pigmentosa cell line contained a naturally occurring mutation of S571 to isoleucine (S571I), which suggests that phosphorylation of S571 may play a role in diseases such as retinitis pigmentosa. The kinase responsible for phosphorylation of S575 was not identified despite investigation of a variety of well known proline directed kinases (e.g. ERK, JNK and CDKs, Figures 3.9, 3.10, 3.11).

GSK3 was originally identified as the third kinase responsible for the phosphorylation of glycogen synthase, which decreased glycogen synthase activity (Embi et al., 1980; Rylatt et al., 1980). 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 (Frame and Cohen, 2001)).

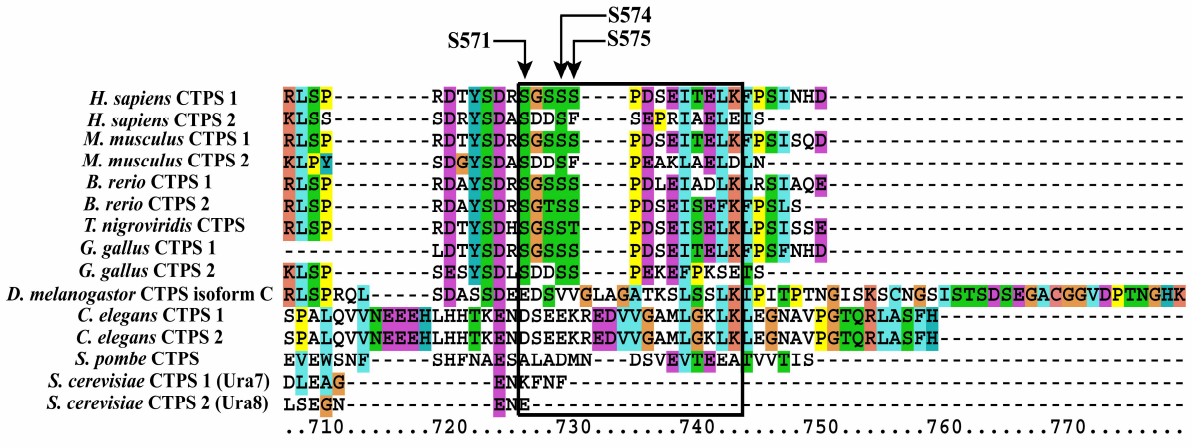


Figure 3.14 Carboxy terminus sequence alignment of CTPS1 and CTPS2 from various organisms.

Clustal X was used to align the carboxy terminus of CTPS1 and CTPS2 from a variety of organisms. S571, S574 and S575 are indicated. Box designates the tryptic peptide identified in this study. The sequences used are as follows (NCBI Accession number): *H. sapiens* CTPS1 (NP_001896), *H. sapiens* CTPS2 (Q9NRF8), *M. musculus* CTPS1 (P70698), *M. musculus* CTPS2 (P70303), *B. rerio* CTPS1 (NP_954681), *B. rerio* CTPS2 (XP_001341091), *T. nigroviridis* CTPS (CAG00263), *G. gallus* CTPS1 (XP_417709), *G. gallus* CTPS2 (Q5F3Z1), *D. melanogastor* CTPS isoform C (NP_730023), *C. elegans* CTPS1 (CAA16517), *C. elegans* CTPS2 (CAD21649), *S. pombe* (CAA15716), *S. cerevisiae* CTPS1 (P28274), *S. cerevisiae* CTPS2 (P38627).

The decrease in CTPS1 activity by GSK3 is consistent with GSK3 negatively regulating the function 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 carboxy-terminus of mammalian CTP: phosphocholine cytidylyl transferase (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 (MacDonald and Kent, 1994; Watkins and Kent, 1991). Moreover CCT activity is decreased by phosphorylation (MacDonald and Kent, 1994; Watkins and Kent, 1991). Multiple phosphorylation sites in the carboxy-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 colleagues demonstrated the critical role of CTP and CTPS phosphorylation in regulating phospholipid synthesis (Choi et al., 2003; McDonough et al., 1995; Park et al., 2003) and Mclarty and Hatch showed CTPS activity is critical for phospholipid synthesis in mammalian cells supporting the notion that CTP synthesis and phospholipid synthesis could be coordinately regulated (Hatch and McClarty, 1996). Whether or not these events are coordinated is an interesting possibility that remains to be explored

Several large-scale analyses of the phosphoproteome have identified numerous phosphorylation sites in the carboxy-terminus of CTPS1 (S562, T566, Y567, S568, S571, S573, S574, S575, S578 and T581) (Olsen et al., 2006). Our studies confirmed the

phosphorylation of S571, S574 and S575. The fact that the other sites were not detected in our analysis suggests that these 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 a anti-phosphotyrosine antibody, but did not to require S571 to be phosphorylated (data not shown). Phosphoaminoacid 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. Phosphorylation of tyrosine was below the limits of detection by radioactivity (data not shown). The observation that multiple phosphorylation sites are clustered in the carboxy-terminus of mammalian CTPS1 suggests that multiple kinases may be involved in phosphorylation regulation of CTPS1, including other hierarchal kinases such as casein kinase 1 (CK I).

Our investigation of CK I demonstrated that CK I was involved in CTPS1 phosphorylation and inhibition of CK I did not affect CTPS1 activity under the conditions tested (Figure 3.12). Further investigation of the phosphorylation and regulation of CTPS1 by CK I is needed before we know how CK I is involved in CTPS1 function. One possibility that remains is that the coordinated action of CK I and GSK3 are necessary for full inhibition of CTPS1.

The crystal structures of bacterial CTPS have identified conserved amino acids that are proposed to be necessary for catalysis, dimerization and tetramerization (Endrizzi et al., 2004; Goto et al., 2004). However, the C-terminal region containing S571, S574 and S575 is not present in these three-dimensional structures making it difficult to predict exactly how phosphorylation of the C-terminus affects CTPS activity.

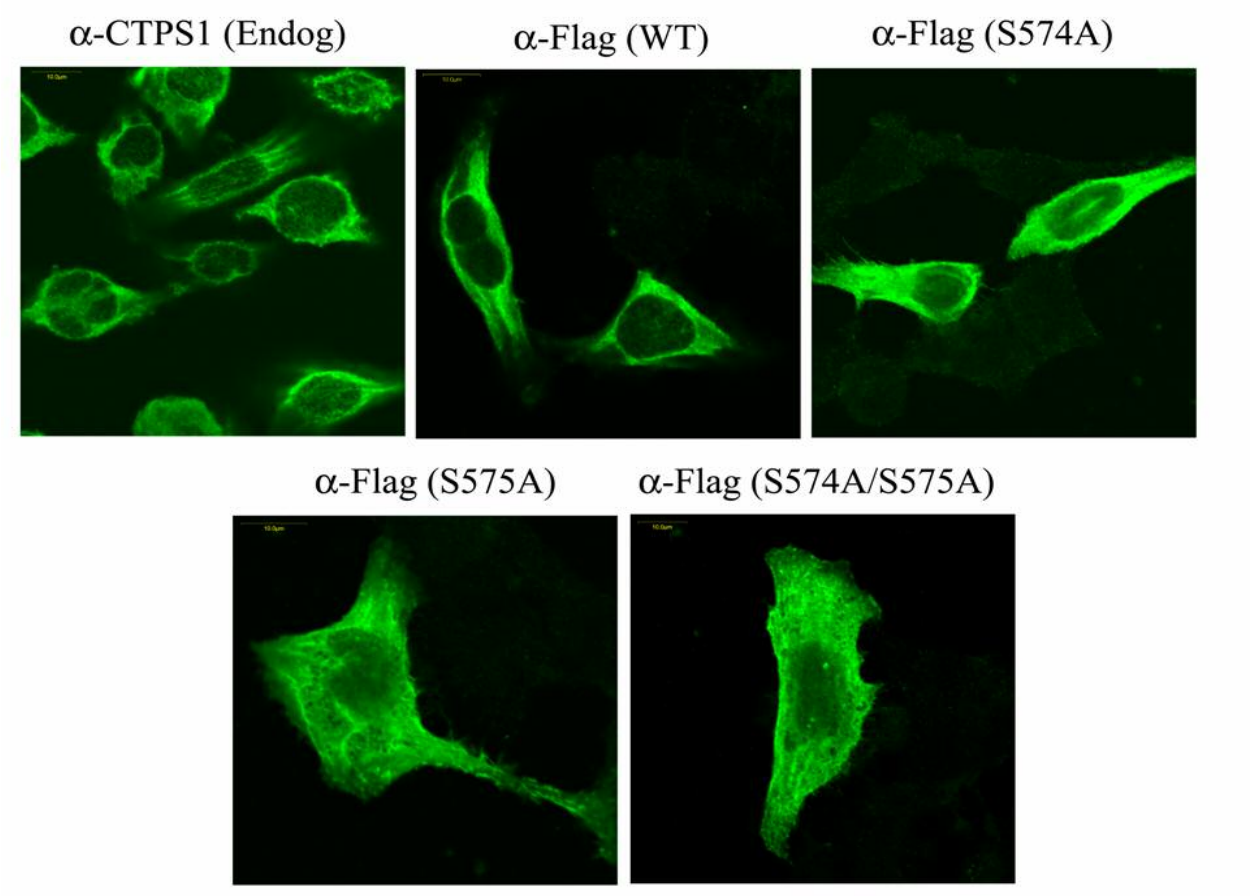


Figure 3.15 Intracellular Localization of Endogenous, WT, S574A, S575A, S574A/S575A CTPS1 in HeLa Cells

HeLa cells were fixed, permeabilized, blocked with horse serum plus BSA and then incubated with primary followed by fluorophore-conjugated secondary antibodies. For staining of endogenous CTPS1, 1:500 dilution in blocking buffer of α-CTPS1 antibody was used. For staining of transfected CTPS1, 1:300 dilution in blocking buffer of α-Flag antibody was used. Secondary antibody for staining of endogenous CTPS1 was goat-anti-rabbit Alexa 488 conjugated (1:1000) and the secondary antibody for staining of Flag-tagged CTPS1 was goat-anti-mouse Alexa 488 conjugated (1:1000) antibody. Cells were visualized using an Olympus confocal microscope. Detailed procedures can be found in "Experimental Procedures".

Analysis of intracellular localization of S574A, S575A and S574A/S575A CTPS1 in HeLa cells did not show a robust change in CTPS1 localization suggesting that phosphorylation does not affect CTPS1 localization under the conditions tested (Figure 3.15).

Analysis of the hydrophobicity/hydrophilicity of human CTPS1 using the Kyte-Doolittle hydrophobicity scale indicates that the C-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 (Pappas et al., 1998) providing a precedent for how phosphorylation of human CTPS1 may regulate CTPS activity. Phosphorylation of human CTPS1 on the C-terminus could directly influence tetramerization (e.g. negative charges of phosphate could cause domains to shift allowing for tetramerization) or phosphorylation of the C-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 could 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 (Meek et al., 2004). .

GSK3 activity is regulated following a variety of stress stimuli, including serum withdrawal/nutrient deprivation, and during development through Wnt and Notch signaling (Welsh et al., 1994) (reviewed in (Marikawa, 2006; Robert and Lallemand, 2006)). Our data suggests that mammalian CTPS1 may be involved in development and/or physiological stresses that involve serum/nutrient withdrawal such as stroke or ischemia (Figure 3.16).

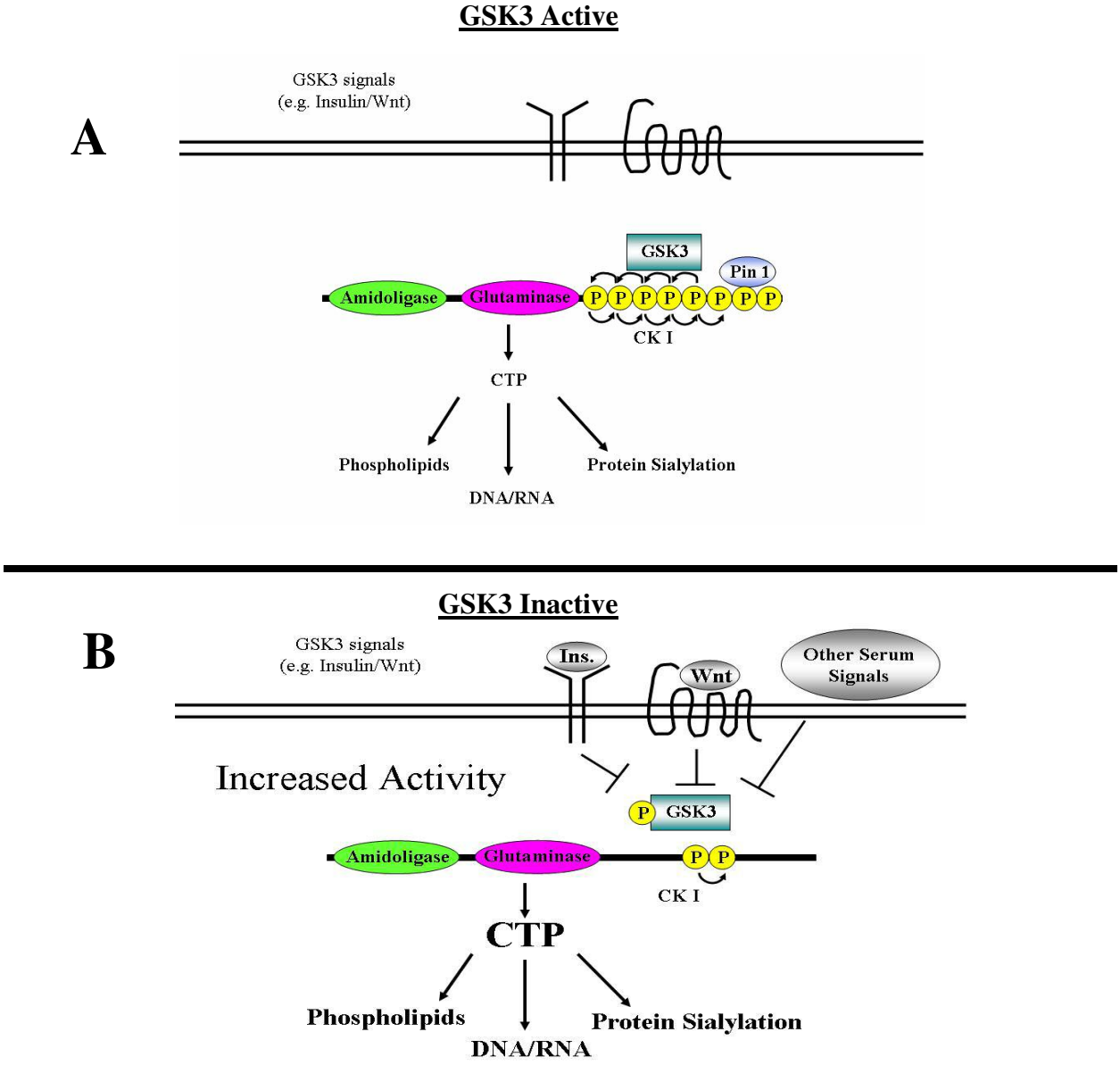


Figure 3.16 Summary Model of CTPS1 Phosphorylation and Possible Impact on CTP-dependent Processes

(A) When GSK3 is active (Ser9 (β) or Ser21 (α) is not phosphorylated) CTPS1 is phosphorylated by GSK3, inhibiting CTPS1 activity and resulting in decreased CTP levels and lower activity of CTP-dependent processes. (B) When GSK3 is inactive (Ser9 (β) or Ser21 (α) is phosphorylated) then CTPS1 is less phosphorylated, in particular S571, resulting

in increased CTPS1 activity ultimately translating into increased output of CTP-dependent processes.

CHAPTER

4. Human CTPS1 Interacting Proteins

4.1 Abstract

Many GSK3 phosphorylation targets are known substrates for the peptidyl prolyl isomerase Pin1, which isomerizes proline bonds juxtaposed to a phosphorylated serine or threonine Since S575 is a phosphorylated residue next to a proline we tested whether Pin1 was a CTPS1 interacting protein using GST-Pin1 binding assays. CTPS1 was found to interact with GST-Pin1 in a S575 dependent manner suggesting prolyl isomerization may regulate CTPS1 function. Since the expression of CTPS1 has not been well characterized, we investigated CTPS1 expression in various rat tissue extracts and cell lines. We found that CTPS1 was most highly expressed in brain and liver from rat tissue and HEK 293 cells expressed the most CTPS1 protein of all the cell lines examined. Differentiation of 3T3 cells to adipocytes decreased CTPS1 protein expression suggesting CTPS1 expression decreased during differentiation. Downregulation of c-myc is necessary for 3T3 differentiation into adipocytes and c-myc has been shown to be required for the expression of the rate-limiting enzyme, CAD, in the *de novo* synthesis of uridine nucleotides (Miltenberger et al., 1995; Pulverer et al., 1994). We therefore, investigated whether c-myc was involved in regulating CTPS1 expression levels. Immunoblotting for CTPS1 showed that CTPS1 protein levels were decreased in c-myc$^{-/-}$ cells compared to c-myc$^{+/+}$ cells. Reintroduction of c-myc into c-myc$^{-/-}$ cells restored CTPS1 expression implicating c-myc in regulating CTPS1 expression.

While investigating CTPS1 subcellular localization we noticed that tubulin and CTPS1 colocalized. Treatment with the microtubule polymerizing agent, taxol, appeared to enhance the colocalization of tubulin and CTPS1. Cotreatment with the CTPS inhibitor, CPEC, and taxol failed to disrupt the colocalization suggesting that the colocalization was not dependent on CTPS activity. Investigation using A10 rat smooth muscle cells demonstrated that CTPS1 and tubulin colocalized more strongly during mitosis at what appeared to be the mitotic spindle. Immunoprecipitation and reciprocal immunoprecipitation verified the interaction between tubulin and CTPS1. A screen to identify other CTPS1 interacting proteins identified thirteen other CTPS1 coimmunoprecipitating proteins, many of which were cytoskeletal. Thus these studies provide novel information into the potential interacting proteins that may regulate CTPS1 function.

4.2 Introduction

In addition to phosphorylation, enzyme activity can be regulated by protein-protein interactions. Although, there have been several large scale analyses of protein-protein interaction networks, these studies relied on ectopically expressed proteins or non-mammalian organisms such as *S. cerevisiae*. As observed with the phosphorylation of CTPS1 (see Chapter 3), mammalian CTPS1 can be regulated differently than CTPS in *S. cerevisiae*. We therefore investigated CTPS1 interacting proteins using endogenously expressed CTPS1 in mammalian cells. Since phospholipid synthesis in mammalian cells has been shown to be localized to distinct subcellular compartments [20-22] and other pyrimidine metabolic enzymes have also been shown to have distinct subcellular distribution (e.g.

uridine phosphorylase with vimentin [23], cytidine deaminase within the nucleus [24]) we predicted that CTPS1 will interact with a unique subset of proteins

4.3 Results

4.3.1 Pin1 Interacts with CTPS1

Pin1 is a peptidyl prolyl isomerase that recognizes phosphorylated serines or threonine N-terminal to prolines. Upon binding a phosphorylated serine or threonine, Pin1 catalyzes the cis to trans isomerization of the prolyl peptide bond ((Lu et al., 1996; Ranganathan et al., 1997). Isomerization of the proline bond in many proteins have been shown to regulate their function. For instance CDK2, MAPKs and the phosphatase PP2A prefer their substrates be in trans configuration for catalysis (Brown et al., 1999; Weiwad et al., 2000; Zhou et al., 2000). Some of the Pin1 targets are known substrates of GSK3 (e.g tau protein) suggesting that CTPS1 might also be a Pin1 target. Using *Xenopus laevis* Pin1 attached to GST, GST-xPin1, HEK 293 cell lysates were used to determine whether endogenous CTPS1 could bind to GST-xPin1. Compared to GST, coimmunoprecipitation experiments demonstrated that GST-xPin1 and CTPS1 formed a complex with each other suggesting that Pin1 and CTPS1 can interact (Figure 4.1). To determine if this complex formation was dependent on S575, GST-xPin1 was incubated with lysate from untransfected HEK 293 cells or HEK 293 cells transfected with WT or S575A flag-tagged mutant. Under these conditions, the WT CTPS1 is known to be phosphorylated on S575 whereas the mutant S575A is not (Chapter 3). The CTPS1 S575A mutant protein showed decreased ability to interact with GST-xPin1 compared to WT-CTPS1 demonstrating that Pin1 binding was dependent on S575 phosphorylation (Figure 4.2). The decreased ability of S575A CTPS1 to interact with Pin1 was not because the GST-xPin1 lost the ability to interact with CTPS1,

because endogenous CTPS1 interacted with GST-xPin1 as shown (Figure 4.2). To determine whether Pin1 binding could alter CTPS1 activity, CTPS1 activity was determined from HEK 293 cells transfected with siRNAs directed towards Pin1 or non-targeting siRNA. Despite significantly decreased protein levels of Pin1 after siRNA treatment, CTPS1 activity was not affected indicating that, under the conditions tested, Pin1 does not alter activity or protein stability of CTPS1 (Figure 4.3). However, further experiments to investigate if Pin1 binding affects CTPS phosphorylation, localization or stability are necessary.

4.3.2 Tubulin is a CTPS1 Interacting Protein

4.3.2.1 Colocalization of Tubulin with CTPS1 in HEK 293 Cells

While investigating the localization of CTPS1 in HEK 293 cells we observed CTPS1 localization suggesting a microtubule specific localization (Figure 4.4). Immunostaining of HEK 293 cells for endogenous CTPS1 and α-tubulin revealed that CTPS1 colocalized with microtubules as observed by confocal microscopy (Figure 4.4).

4.3.2.2 Colocalization of Tubulin with CTPS1 in Taxol-treated HEK 293 Cells

In an attempt to increase the incidence of observing the colocalization of microtubules and CTPS1, HEK 293 cells were treated with the microtubule stabilizing agent, taxol (100 nM, 7 hours). As seen in Figure 4.5, taxol induced the formation of "tufts" of microtubules and CTPS1 colocalized with these tufts, indicating that under conditions when microtubules are induced to polymerize, the colocalization of CTPS1 and microtubules is enhanced.

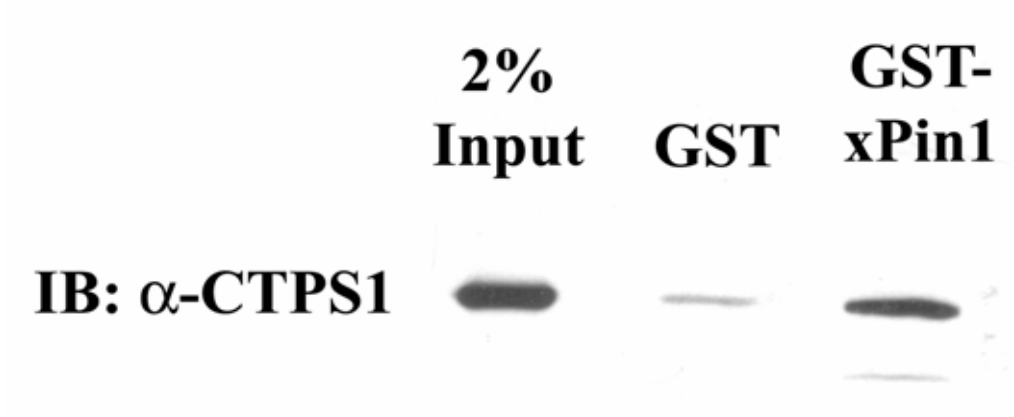


Figure 4.1 Endogenous CTPS1 can Interact with GST-xPin1

HEK 293 cells were lysed as described in "Experimental Procedures" and incubated with approximately 20 µg of GST or GST-xPin1. 2% of the total amount of lysate incubated with the GST fusion proteins is shown as a comparison. Affinity-precipitated proteins were washed with lysis buffer, separated by SDS-PAGE and analyzed by immunoblotting for CTPS1.

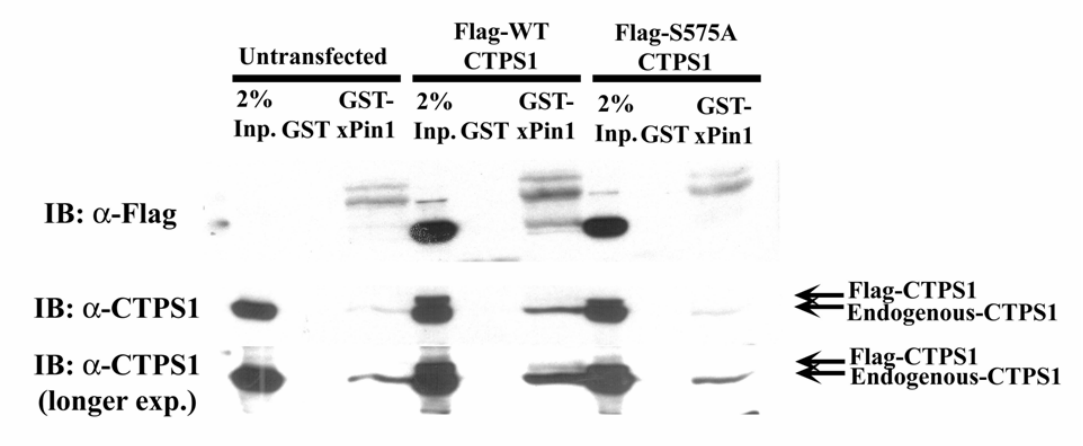


Figure 4.2 S575 is Involved in Pin1 Interacting with CTPS1

HEK 293 cell lysates and analysis was performed as described in the legend to Figure 4.1. Immunoblot analysis involved probing for the Flag tag on transfected CTPS1 where indicated. Endogenous CTPS1 was immunoblotted for comparison using an antibody specific for this protein.

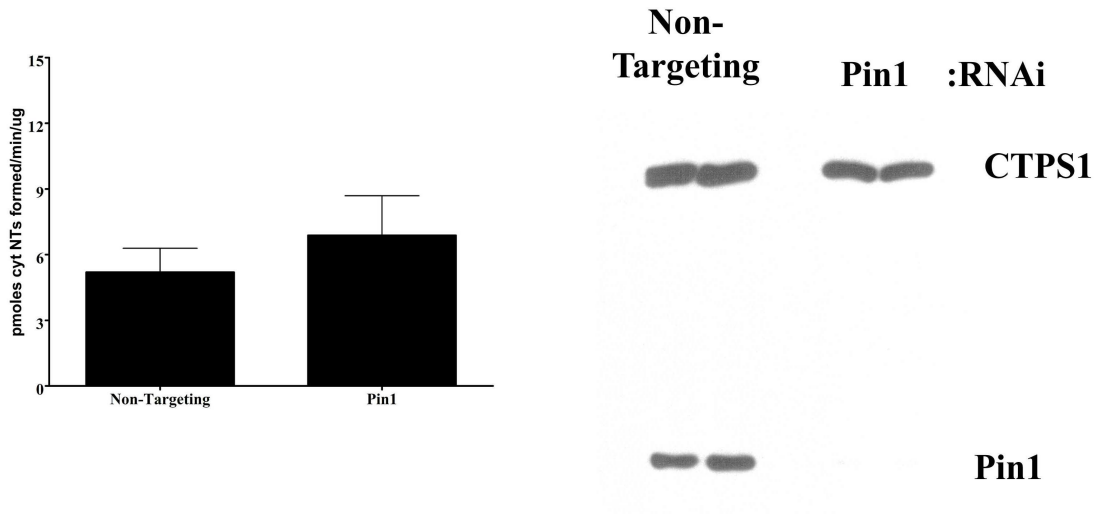


Figure 4.3 Silencing Pin1 Expression with siRNA does not Affect CTPS1 Activity

HEK 293 cells were transfected with Smart Pool™ siRNA per manufacturers instructions, maintained in 0.1% FBS overnight and activity of CTPS1 was assessed as described in "Experimental Procedures". Expression of Pin1 and CTPS1 was assessed by immunoblot as described in "Experimental Procedures".

4.3.2.3 Effect of CPEC and CPEC plus Taxol on Tubulin and CTPS1 Localization

To determine if inhibition of CTPS affected microtubules, HEK 293 cells were treated with CPEC, a highly specific inhibitor of CTPS. Treatment with CPEC appeared to increase the size of cell nuclei and also appeared to increase the network of microtubules (Figure 4.6). Since CPEC can cause cell cycle arrest (Huang et al., 2004), the effects observed on microtubules may be an indirect cause of inhibition of these cellular processes or disruption of other CTP-requiring processes.

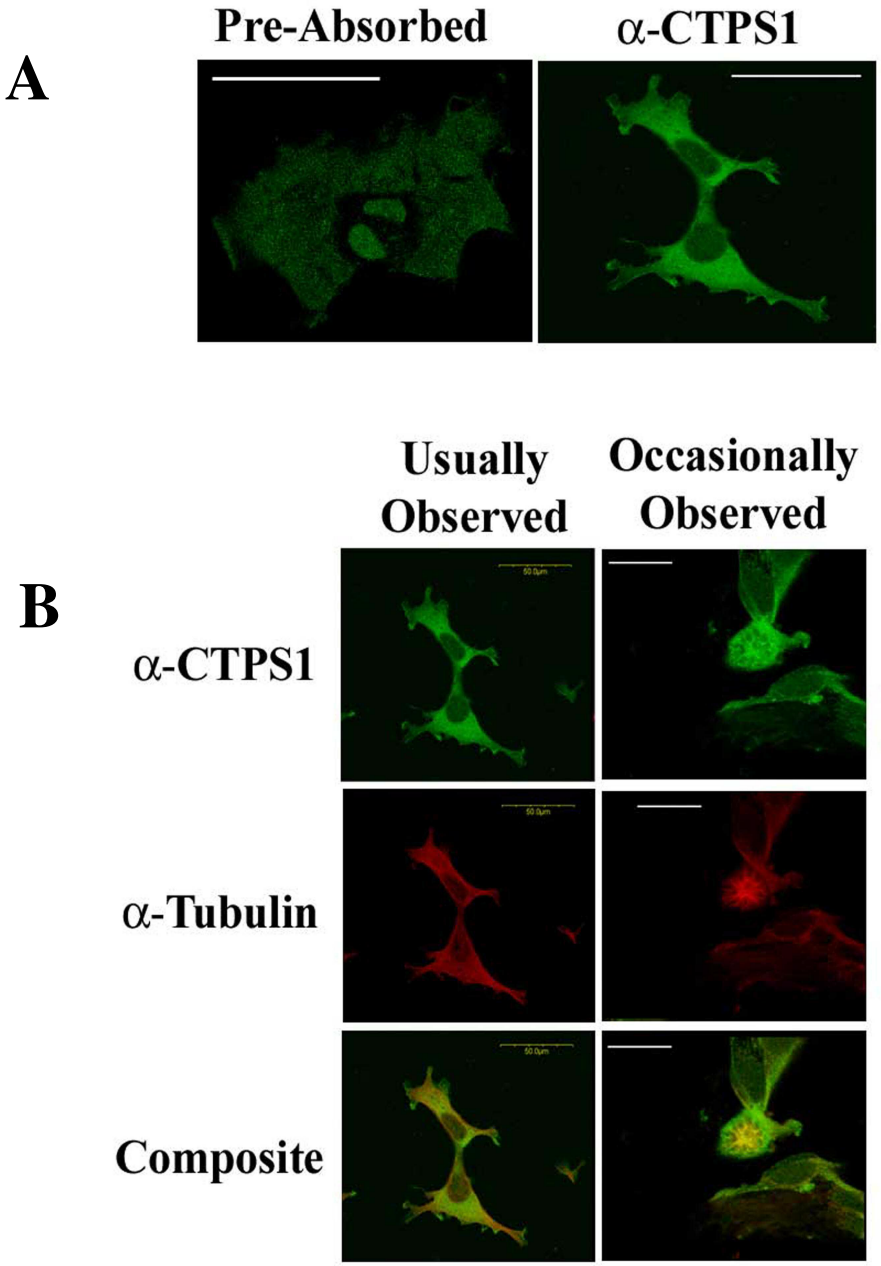


Figure 4.4 Immunostaining of CTPS1 and Tubulin in Unstimulated HEK 293 Cells

(A) HEK 293 cells were immunostained with α-CTPS1 antibody or α-CTPS1 antibody following preabsorption with the antigenic peptide as described in "Experimental Procedures". (B) Shown are typical immunostaining of CTPS1 and tubulin in most HEK 293 cells ("Usually Observed") and immunostaining observed occasionally in some HEK 293 cells. Immunostaining of CTPS1 and tubulin was done as described in "Experimental Procedures" using Alexa 488 conjugated secondary for staining of CTPS1 and Alexa 594 conjugated secondary for staining anti-tubulin.

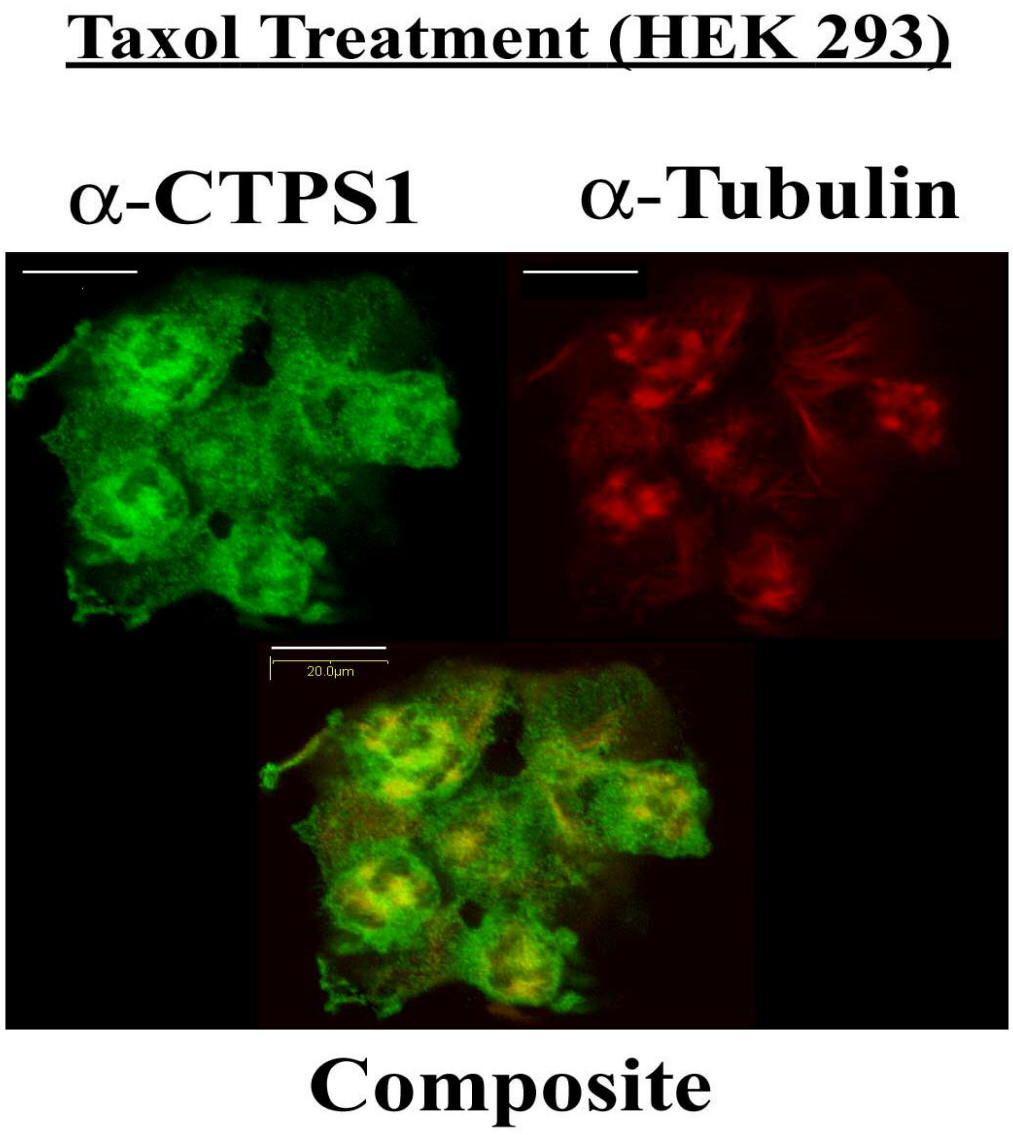


Figure 4.5 CTPS1 and Tubulin Immunostaining of Taxol-treated HEK 293 Cells

HEK 293 cells were treated with 250 nM taxol for 7 hours prior to fixing cells. Immunostaining of HEK 293 cells for CTPS1 and tubulin was done as described in "Experimental Procedures" and legend to figure 4.4.

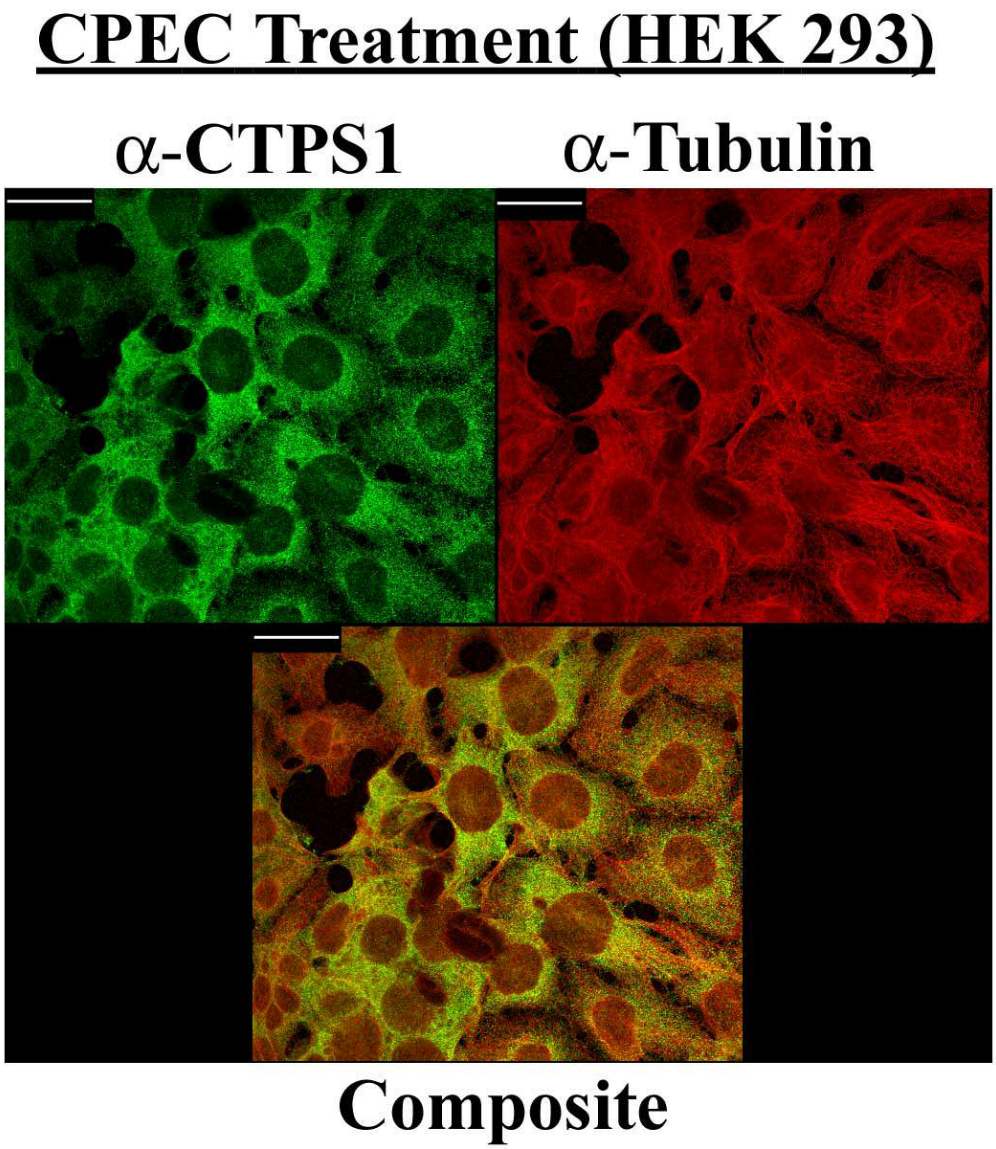


Figure 4.6 Immunostaining of CTPS1 and Tubulin in CPEC-treated HEK 293 cells

HEK 293 cells were treated with 100 nM CPEC for 48 hours prior to fixing cells and immunostaining for CTPS1 and tubulin as described in "Experimental Procedures" and the legend to figure 4.4.

We also investigated whether cotreatment with CPEC and taxol would alter the microtubule architecture and/or CTPS1 colocalization. The cotreatment of HEK 293 cells with CPEC plus taxol induced the "tufts" of microtubules to form. In cells where "tufts" were not observed, the microtubule network seemed to be surrounding the enlarged nucleus (Figure 4.7). CTPS1 colocalized with "tufts" of microtubules in the presence of CPEC plus taxol and appeared to colocalize with the microtubule network surrounding the nucleus in cells where the "tufts" were absent (Figure 4.7). The fact that CPEC did not prevent colocalization of CTPS1 with taxol-induced microtubule "tufts" indicates that CTPS activity was not required for this colocalization.

4.3.2.4 Colocalization of CTPS1 and Tubulin in Mitotic A10 Rat Smooth Muscle Cells

To determine whether the CTPS1 colocalization with polymerized microtubules occurs in a cell cycle-dependent manner, A10 rat smooth muscle cells were immunostained for CTPS1 and α-tubulin and cells in interphase and mitosis were visualized. As observed in Figure 4.8, CTPS1 localized to the cell periphery and areas surrounding the nucleus in interphase and was not specifically interacting with all the microtubules. However, in mitosis CTPS1 strongly colocalized with microtubules involved in the formation of the mitotic spindle, indicating that in mitosis CTPS1 may have a mitotic function involving microtubules.

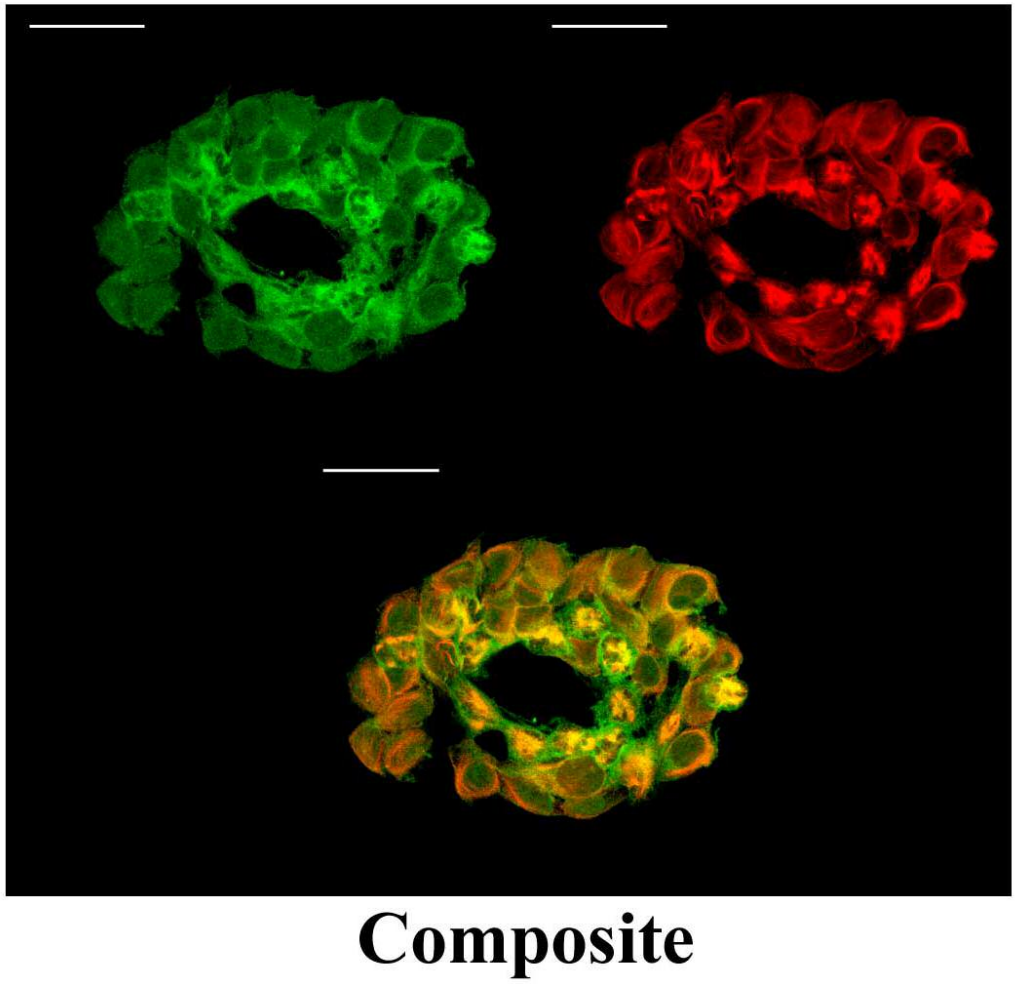


Figure 4.7 Immunostaining of CTPS1 and Tubulin in HEK 293 Cells Treated with CPEC and Taxol

HEK 293 cells were treated with 100 nM CPEC and 250 nM taxol for 7 hours prior to fixing cells for immunostaining. CTPS1 and tubulin immunostaining were done as described in "Experimental Procedures" and the legend to figure 4.4.

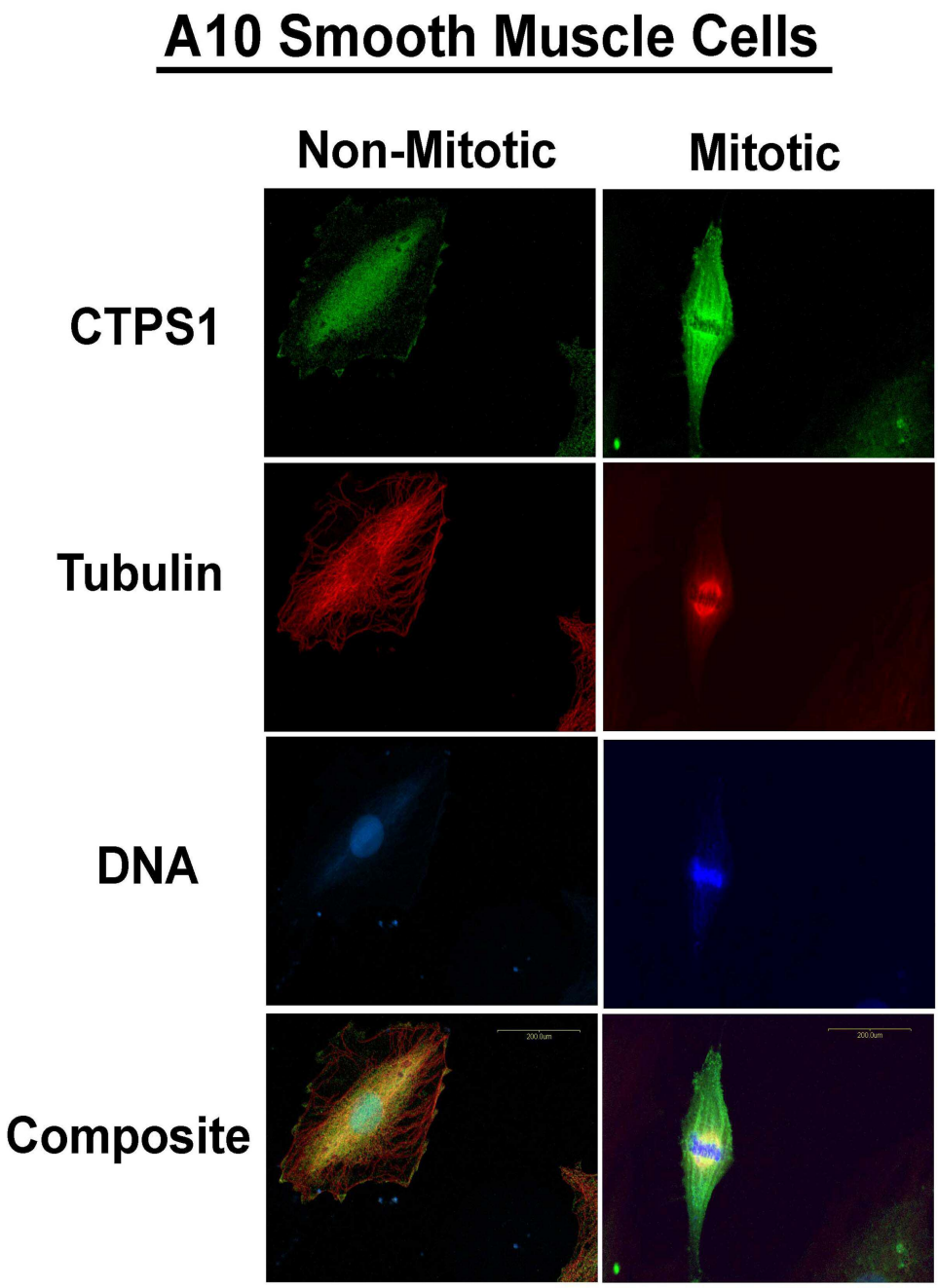


Figure 4.8 Immunostaining of CTPS1 and Tubulin in Interphase and Mitotic A10 Smooth Muscle Cells

A10 rat aortic smooth muscle cells were grown and immunostained as described in "Experimental Procedures" and in the legend to figure 4.4. Shown are a representative cell in interphase and a cell in mitosis.

4.3.2.5 Immunoprecipitation and Reciprocal Immunoprecipitation of CTPS1 and Tubulin

To verify that the colocalization of CTPS1 and tubulin occurred through complex formation of the two proteins, CTPS1 or tubulin was immunoprecipitated and the reciprocal protein was immunoblotted for. Immunoprecipitation of CTPS1 from HEK 293 cells showed that tubulin coimmunoprecipitated with CTPS1. Preabsorbing the α-CTPS1 antibody with the antigenic peptide abrogated this coimmunoprecipitation, indicating this interaction was CTPS1 dependent (Figure 4.9). Reciprocally, CTPS1 was found to coimmunoprecipitate with α-tubulin, but not with protein-A agarose alone, indicating that the interaction of immunoprecipitated CTPS1 was α-tubulin dependent (Figure 4.9).

4.3.3 Screen for Other CTPS1 Interacting Proteins

The following strategy was used to identify other CTPS1 interacting proteins: 1) immunoprecipitating CTPS1 from HEK 293 cells using the CTPS1 antibody preabsorbed with or without the antigenic peptide; 2) separating coimmunoprecipitating proteins by SDS PAGE; 3) radiolabeling cells with [^{35}S]methionine to probe for interactions radiolabeled proteins; 4) identifying putative interacting proteins by mass spectrometry.

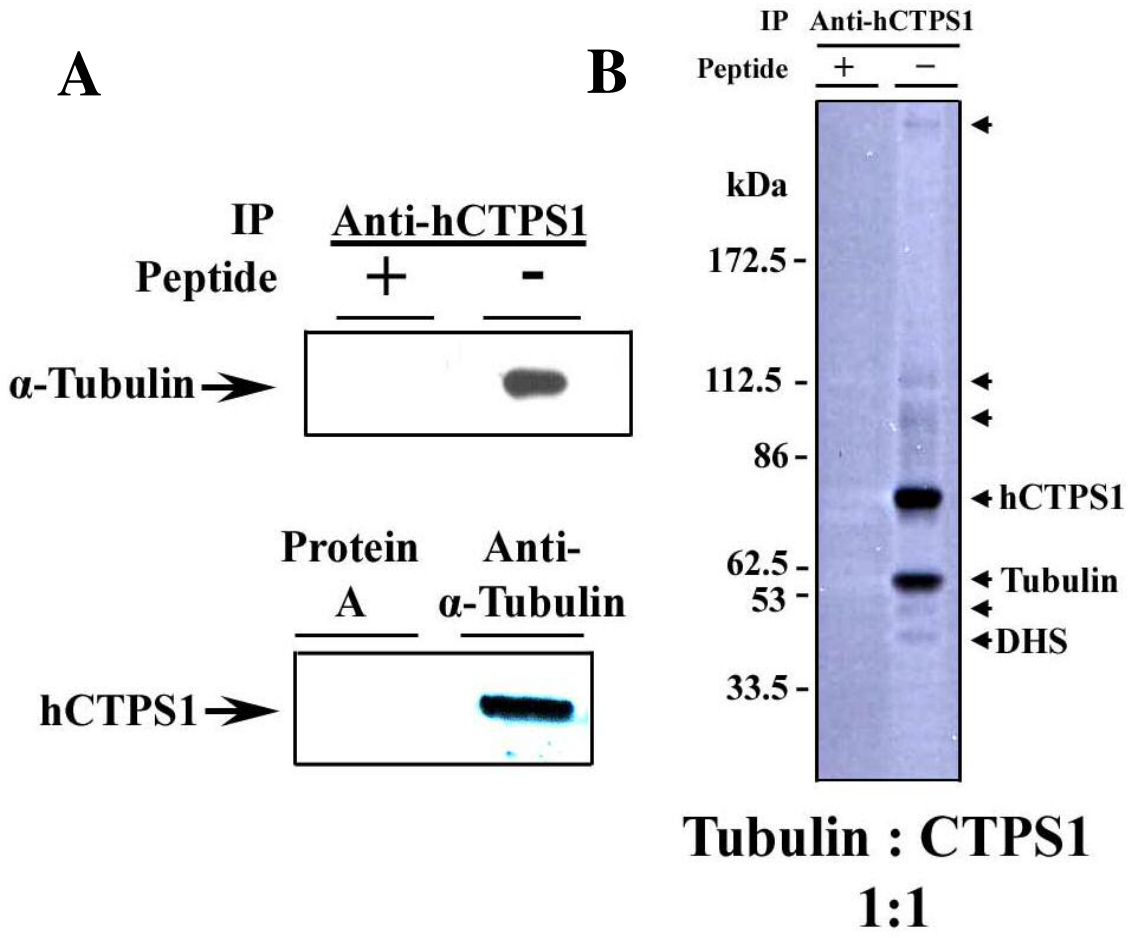


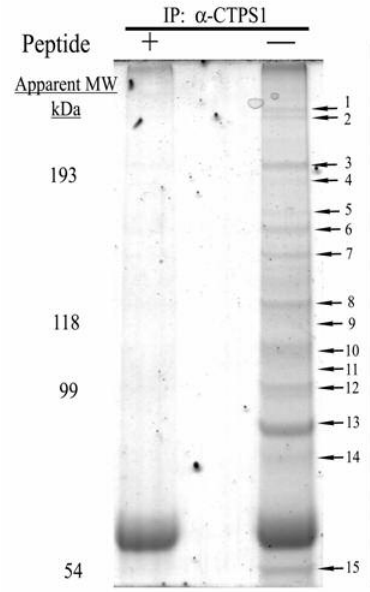
Figure 4.9 Immunoprecipitation and Reciprocal Immunoprecipitation of CTPS1 and Tubulin. Identification of CTPS1 Interacting Proteins by [^{35}S]Methionine Labeling

(A) Anti-CTPS1 antibody or anti-CTPS1 antibody preabsorbed with antigenic peptide was used to immunoprecipitate CTPS1 from HEK 293 cells as described previously. Coimmunoprecipitating proteins were separated by SDS PAGE and α-tubulin was visualized by immunoblot analysis as described in "Experimental Procedures". Anti-α-tubulin antibody or protein A alone was used to immunoprecipitate α-tubulin from HEK 293 cell lysates as described in "Experimental Procedures". Coimmunoprecipitating proteins were separated by SDS PAGE and CTPS1 was visualized by immunoblotting for CTPS1. (B) HEK293 cells were labeled overnight with [^{35}S]methionine and immunoprecipitated (IP) the following day with anti-CTPS1 antibody or anti-CTPS1 antibody preabsorbed with antigenic peptide. Immunoprecipitates were separated on a 4-12% acrylamide gel. CTPS1 co-immunoprecipitating proteins visualized on autoradiograph of dried gel were identified by MALDI-TOF mass spectrometry.

Initially we labeled HEK 293 cells with [^{35}S]methionine to enhance detection of CTPS1 coimmunoprecipitating proteins. This had the unexpected advantage of labeling a protein that comigrated on SDS PAGE with the rabbit IgG heavy chain, thereby allowing us to identify this protein by mass spectrometry. Specifically this was accomplished by gating the peptide search program to search the human database only (i.e. did not include the rabbit database since this would undoubtedly identify IgG). This protein was identified as tubulin (peptides identifying both α and β tubulin were found), and since tubulin and CTPS1 were both labeled with [S^{35}]methionine, the molar stoichiometry between tubulin and CTPS1 was estimated to be 1:1. This data strongly suggested that CTPS1 formed a complex with tubulin and supported our earlier observations. An additional protein identified by this method was deoxyhypusine synthase, DHS (Figure 4.9).

Further investigation using the same strategy without [^{35}S]methionine, but using colloidal coomassie staining of the SDS PAGE gel, revealed thirteen coimmunoprecipitating proteins (Figure 4.10). Included were the rate-limiting enzyme for the *de novo* synthesis of uridine pyrimidines, CAD, and many cytoskeletal proteins. The identification of CAD subunit interacting with CTPS1 in yeast has been observed in a large scale analysis of the yeast interactome (Gavin et al., 2002) and the number of cytoskeletal proteins coimmunoprecipitating with CTPS1 is not unexpected due to the interaction of CTPS1 with tubulin. Interestingly, retinitis pigmentosa like 1 protein (RPL1) was identified as a CTPS1 coimmunoprecipitating protein. The apparent molecular weight (~55 kDa) of RPL1 is significantly smaller than the predicted molecular weight (~200 kDa), which may indicate this protein was proteolyzed. The mutation of the GSK3 phosphorylation site in CTPS1, S571 (See chapter 3), was from a clone derived from a retinitis pigmentosa cell line

suggesting CTPS1 may play a role in retinitis pigmentosa. Further research is required to explore this interesting possibility.



Band Number	Protein Name	Accession Number	Protein Score
1	Cytoplasmic Dynein HC	Q14204	399
2	DNA Dependent Protein Kinase Catalytic Subunit	P78527	278
3	Filamin A	P21333	159
4	Fatty Acid Synthase GCN-like Protein CAD	P49327 Q92616 P27708	247 150 128
5	Pericentrin IQGAP p195	O95613 P46940	96 91
6	Bifunctional t-RNA Synthetase Glutamyl Hemoglobin-haptoglobin binding protein A	P07814 Q48153	155 115
7	Isoleucyl t-RNA Synthetase Spectrin	P41252 P13395	117 101
8	Spectrin	Q00963	101
9	?		
10	Spectrin Golgin Subfamily A Member 4	Q9NRC6 Q13439	113 101
11	Microtubule/Actin Cross-linking Factor	QUPN3	127
12	Nucleopore Complex Protein Nup93 HSP 90	Q8N1F7 P08238	94 94
13	CTP Synthetase 1	P17812	140
14	?		
15	Retinitis Pigmentosa (mouse) Microtubule/Actin Cross-linking Factor	Q8CGM2 Q96PK2	134 109

Figure 4.10 Identification of CTPS1 Interacting Proteins (Colloidal Coomassie Stain) by Mass Spectrometry

CTPS1 was immunoprecipitated with anti-CTPS1 antibody or anti-CTPS1 antibody preabsorbed with antigenic peptide as described in legend for Figure 4.9. Indicated coomassie stained protein bands were excised, trypsin digested and identified by peptide mass fingerprinting (PMF) on a ABI 4700 MALDI TOF/TOF.

4.3.4 CTPS1 Expression in Extracts from Rat Tissue and Lysates from Cell Lines

Since many of the proposed interacting proteins, most notably tubulin, can be found in various cell types, it was important to determine in which cell and/or tissue types CTPS1 was expressed to more fully understand how the identified CTPS1 interacting proteins could influence CTPS1 function.

4.3.4.1 CTPS1 Expression in Rat Tissue Extract

Evidence from studies in various cell types including tumor cells has demonstrated that CTPS activity is increased in rapidly proliferating cells suggesting that CTPS1 may not be highly expressed in post-mitotic differentiated cells. To investigate CTPS1 protein expression, rat tissue was harvested from an adult rat, lysed and homogenized, proteins separated by SDS PAGE and CTPS1 was detected by immunoblotting. Unexpectedly, CTPS1 protein expression in adult rat was highest in brain, a tissue composed of mostly post-mitotic cells, followed by liver (Figure 4.11A). Interestingly microtubules, Pin1 and GSK3 are known to play significant roles in neurons suggesting that these proteins may regulate CTPS1 in brain. Further studies will be needed to determine in which neuronal cell types and brain regions CTPS1 is expressed.

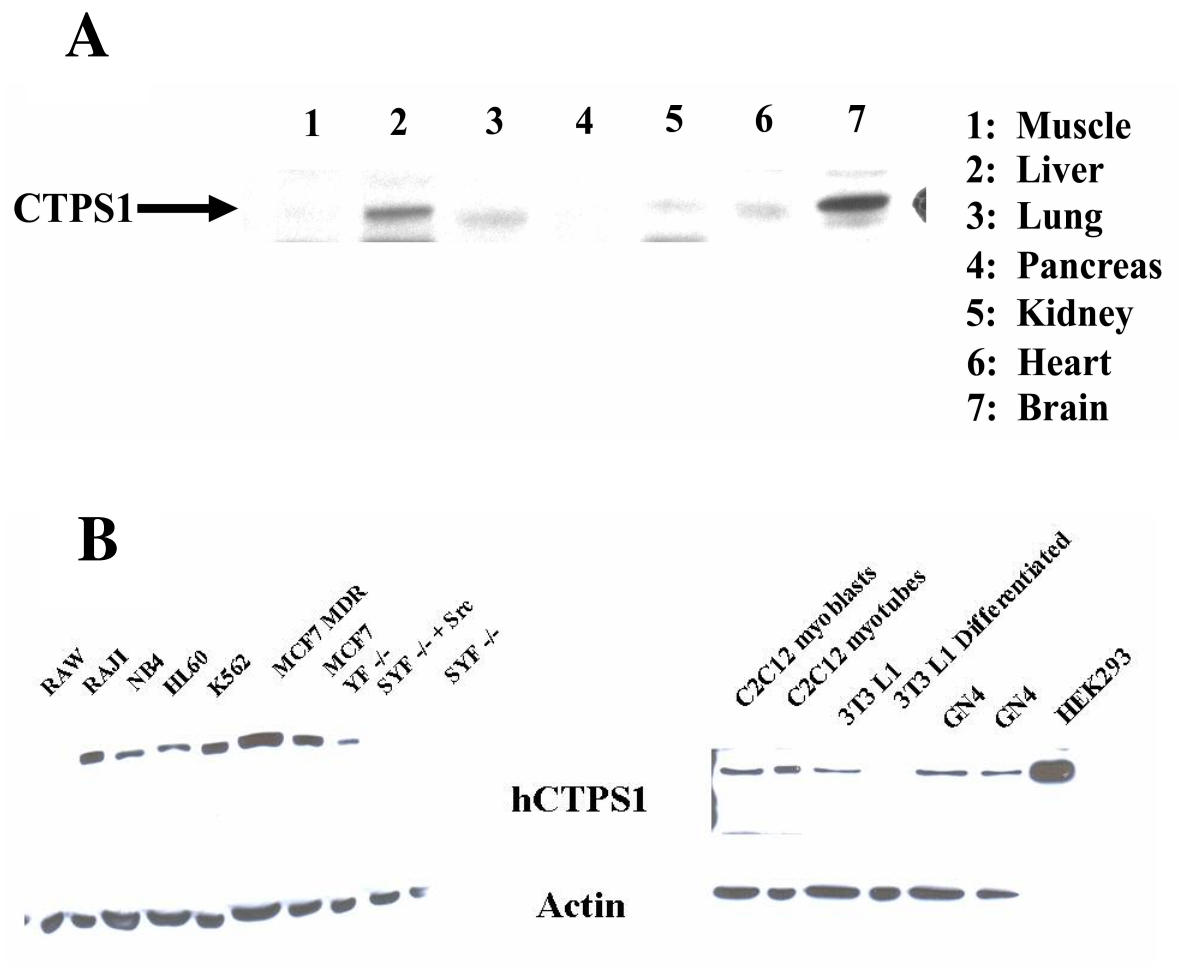


Figure 4.11 CTPS1 Expression in Rat Tissue and Cell Lines

(A) 3 grams of indicated rat tissue was homogenized in SDS RIPA lysis buffer and clarified by centrifugation. 30 µg of protein was separated on a 4-12% gradient gel, transferred to PVDF and immunoblotted for CTPS1. Because the contribution of proteins (e.g. tubulin, actin) normally used to verify equal protein loading vary from tissue to tissue with respect to the total amount of protein, they cannot be used to determine equal loading (B) Cells were lysed in RIPA lysis buffer without SDS and lysates were clarified by centrifugation. 30 µg of protein was separated by SDS PAGE and CTPS1 was visualized by immunoblotting with anti-CTPS1 antibody. β-actin was analyzed as a loading control. RAW = RAW 264.7 murine macrophage cell line, RAJI = Human Burkitt's Lymphoma cell line, NB4 = Human Acute Promyelocytic Leukemia Cell line, HL60 = Human Myeloid Leukemia, K562 = Human Chronic Myelogenous Leukemia, MCF7 MDR = Human Breast Adenocarcinoma cell line-multi-drug resistant, MCF7 = Human Breast Adenocarcinoma cell line, YF-/- = Yes and Fyn kinase knockout murine embryonic fibroblasts, SYF-/- +Src = Src,

Yes and Fyn kinase knockout murine embryonic fibroblasts with Src retransfected back, SYF-/- = Src, Yes, and Fyn knockout murine embryonic fibroblasts, C2C12 myoblasts = Subclone of a murine myoblast cell, C2C12 myotubues = Differentiated C2C12 myoblasts, 3T3 L1 = Substrain of Swiss 3T3 cells, preadipocyte fibroblast-like cell line, 3T3 L1 Differentiated = 3T3 L1 induced to differentiate into adipocytes, GN4 = Rat liver epithelial liver cells, HEK 293 = Human embryonic kidney cells.

4.3.4.2 CTPS1 Expression in Lysates from Various Cell Lines

Investigation of CTPS1 expression of various cell types revealed that HEK 293 cells had the highest CTPS1 expression (far higher than any other cell line tested) indicating HEK 293 cells are a good source for endogenous CTPS1 for use in biochemical studies. Differentiation of C2C12 myoblasts to myotubes did not greatly alter CTPS1 expression. Surprisingly, differentiation of 3T3 L1 cells into adipocytes decreased expression of CTPS1 suggesting that CTPS1 may be important in adipogenesis (Figure 4.11 B).

4.3.5 c-Myc is Involved in Regulating CTPS1 Expression

One of the transcription factors known to regulate 3T3 differentiation into adipocytes and to regulate the transcription of another important pyrimidine biosynthetic enzyme (CAD), is the transcription factor c-myc (Miltenberger et al., 1995; Pulverer et al., 1994). To investigate whether c-myc was involved in the regulation of CTPS1 expression, CTPS1 protein levels was assessed by immunoblotting CTPS1 from c-myc$^{-/-}$ or c-myc$^{+/+}$ MEFs infected with GFP or c-Myc adenovirus in the presence or absence of serum. CTPS1 protein expression was found to be less in the c-myc$^{-/-}$ cells compared to the c-myc $^{+/+}$ cells and the introduction of c-myc by viral infection into the c-myc$^{-/-}$ cells restored CTPS1 expression (Figure 4.12). These results indicate that c-myc can regulate CTPS1 protein expression. Inspection of the promoter sequence of CTPS1 revealed an E-box, c-myc binding site, -70 and -300 bps from the translational start site (See Chapter 1).

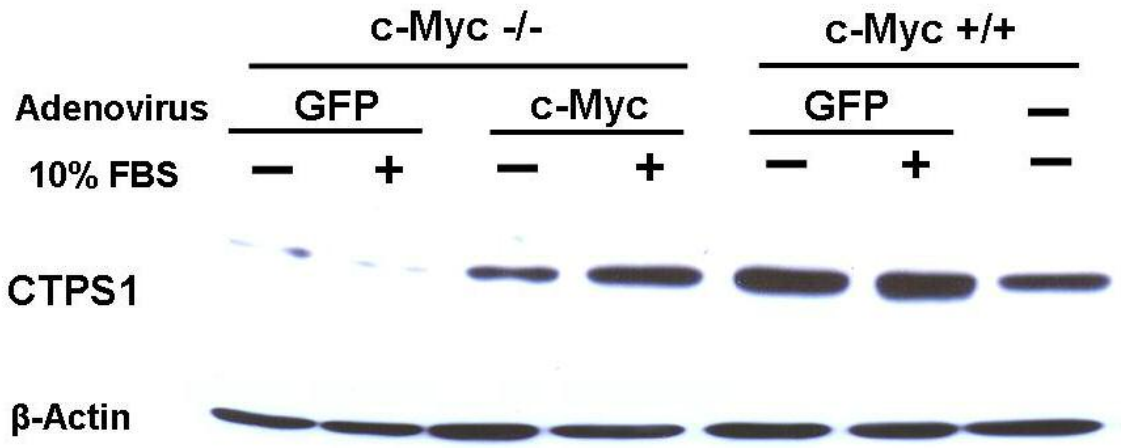


Figure 4.12 Expression of CTPS1 in MEFs from c-myc$^{-/-}$, c-myc$^{+/+}$ and c-myc$^{-/-}$ (addback c-myc) Cells

The indicated MEF were infected with the indicated adenovirus and then media switched to 10% or 0.1% FBS as described in "Experimental Procedures". CTPS1 and β-actin was visualized as described in "Experimental Procedures".

4.4 Conclusion

4.4.1 Pin1 Interaction

Our data indicate that Pin1 could be a novel CTPS1 interacting protein and may regulate CTPS1 function. Under the conditions tested, siRNA against Pin1 did not affect CTPS1 activity indicating that if Pin1 is truly a CTPS1 interacting protein it may regulate CTPS1 activity under conditions different from those tested or Pin1 could regulate other aspects of CTPS1 regulation such as localization or interaction with other proteins. Attempts to immunoprecipitate endogenous Pin1 or endogenous CTPS1 and immunoblot for the reciprocal protein were unsuccessful despite proper immunoprecipitation of the intended protein (data not shown). This may be dependent on the stoichiometry of S575 phosphorylation as our results demonstrated this was required for interaction. The inability to coimmunoprecipitate Pin1 or CTPS1 may be the result of transient interaction between the two proteins. Using a cross-linking agent may help in stabilizing the interaction allowing for verification of this interaction among endogenous proteins. Alternatively, the interaction of the endogenous proteins may be difficult to detect because the Pin1 and CTPS1 interaction is a small portion of the total Pin1 or CTPS1. It is also possible that the Pin1 and CTPS1 do not normally interact, but this seems unlikely given that the interaction depends on a known phosphorylation site (S575) proposed to be important for phosphorylation regulation of CTPS1 (e.g. phosho-S575 primes for GSK3 phosphorylation of S571).

4.4.2 Tubulin Interaction with CTPS1

The tubulin interaction with CTPS1 was unanticipated and little precedent exists for this interaction. One possible reason for this interaction is that tubulin is acting as a scaffold protein, channeling products from one reaction to the next. One of the potentially important tubulin interacting proteins is nucleoside diphosphate (NDP) kinase (Nickerson and Wells, 1984). NDP kinase forms UTP and GTP from UDP and GDP, respectively. Since UTP and GTP are used by CTPS it is possible that polymerized microtubules are acting as a scaffold to allow for more efficient shuttling of NDP products, UTP and GTP, into the CTPS reaction. Another nucleotide enzyme, ribonucleotide reductase, is also involved in microtubule dynamics (Takada et al., 2000) and cytoplasmic dynein, which was identified in our screen of CTPS1 interacting proteins, can hydrolyze CTP faster than ATP (Collins and Vallee, 1989; Shpetner et al., 1988). This further suggests that substrate channeling may be involved in the synthesis of dCDP since CDP is a substrate for ribonucleotide reductase and cytoplasmic dynein would efficiently generate CDP (Figure 4.13).

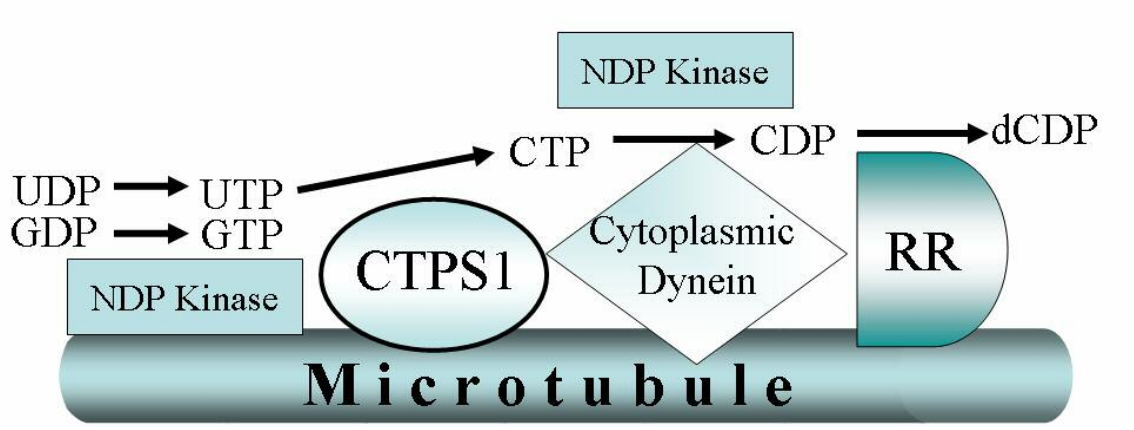


Figure 4.13 Model of Substrate Channeling on Microtubules of Cytidine Nucleotides

NDP kinase can convert GDP and UDP to GTP and UTP, respectively. CTPS can use the GTP as an allosteric activator for forming CTP from the UTP formed in the NDP kinase reaction. Cytoplasmic dynein or NDP kinase (the affinity of CTP for NDP kinase is low, suggesting cytoplasmic dynein as the major enzyme for the formation of CDP) can hydrolyze CTP to form CDP. CDP is an efficient substrate for reduction by ribonucleotide reductase, yielding dCDP, which can be phosphorylated to dCTP for use in DNA synthesis.

4.4.3 CTPS1 Expression in Tissue and Cell Lines

The expression of CTPS1 in rat tissue revealed some unexpected findings and indicates that CTPS1 is expressed in the brain, suggesting a role for CTPS1 in brain function. Interestingly, the ability of CTPS1 to interact with tubulin and Pin1 and to be regulated by GSK3 phosphorylation suggests that CTPS1 may be colocalized with these proteins in this tissue. Furthermore, CTPS1 mRNA is expressed areas in discrete areas zebra fish embryos that give rise to brain structures implicating a role for CTPS1 in neurological development (http://www.zfin.org). Whether or not CTPS1 has a neuronal specific function or dysregulation of CTPS contributes to the development of neurological disorders such as Alzheimers, remains to be investigated.

4.4.4 CTPS1 Expression in Cell Lines

Expression of CTPS1 in cell lines revealed that HEK 293 cells highly express CTPS1 compared to the other cell lines examined. Additionally, CTPS1 protein expression levels were decreased in 3T3 cells stimulated to differentiate into adipocytes indicating a possible role for CTPS1 in adipogenesis.

The observation that CTPS1 protein levels decreased in differentiated 3T3 cells and the fact that c-myc is involved in adipocyte differentiation suggested that CTPS1 may also be regulated by c-myc. In collaboration with Eric M Wauson, we found that c-myc$^{-/-}$ MEFs had decreased CTPS1 protein levels and infection with the adenovirus containing c-myc $^{+/+}$ restored CTPS1 protein expression levels indicating that c-myc is involved in CTPS1 expression. Further investigation will be needed to determine whether c-myc regulates transcription of CTPS1 directly or if the effect of c-myc on CTPS1 protein expression is

indirect. Furthermore, it would be interesting to investigate whether reduction in CTPS1 protein expression is required for 3T3 adipocyte differentiation.

CHAPTER

5. Summary and Future Directions

5.1 Summary

CTP is an essential metabolite required for phospholipid synthesis, protein sialylation and DNA/RNA synthesis. CTPS is a critical regulator of CTP levels in cells and CTPS activity can affect CTP-dependent processes, such as phospholipid synthesis. CTPS can bind four nucleotides (ATP, UTP, CTP and GTP) and the amino acid glutamine, providing exquisite metabolite control of CTPS. In addition to metabolite control, CTPS1 in *S. cerevisiae* (URA7) was found to be regulated by PKA and PKC phosphorylation. This study investigated whether the mammalian CTPS1 is regulated by phosphorylation and if so, what are the kinases responsible. Additionally, we identified hCTPS1 interacting proteins, which may further our understanding of how hCTPS1 is regulated. We found that PKA and PKC are not essential for the phosphorylation of endogenous hCTPS1. However, this result may not be terribly surprising because regulation of nucleotide and lipid metabolism in yeast is expected to be quite different from mammals, which require highly regulated and localized production of these intermediates during different stages of development. Unexpectedly, we found that endogenous hCTPS1 was phosphorylated under low-serum conditions in a GSK3-dependent manner. S571, located in the c-terminus of hCTPS1, was identified as the major GSK phosphorylation site and required S575 to be phosphorylated for this phosphorylation to occur. Incubation of HEK 293 cells in low-serum reduced CTPS1 activity and inhibition of

GSK3 prevented this decrease in CTPS1 activity, indicating that GSK3 phosphorylation of hCTPS1 is inhibitory. Dephosphorylation of hCTPS1 *in vitro* with alkaline phosphatase increased hCTPS1 activity in a time-dependent manner demonstrating that phosphorylation of hCTPS1 is inhibitory. A large-scale analysis of phosphorylation sites in HeLa cells identified multiple phosphorylated residues in the c-terminus of hCTPS1, many of which were also consensus sites for phosphorylation by CK I. Inhibition of CK I demonstrated that CK I is involved in hCTPS1 phosphorylation, but whether this is a direct phosphorylation and which sites are phosphorylated by CK I remains to be determined. Since several GSK3 substrates (e.g. tau protein) can interact with the peptidylprolyl isomerase, Pin1, we investigated whether Pin1 can also interact with hCTPS1. We found that Pin1 can interact with hCTPS1 in a phosphorylation S575-dependent manner. However, reduction of Pin1 expression by siRNA did not alter hCTPS1 activity under the conditions examined, suggesting that Pin1 may regulate a different aspect of hCTPS1 function or regulates hCTPS1 activity under different conditions. Investigation of hCTPS1 interacting proteins also revealed that tubulin is a hCTPS1 interacting protein. The hCTPS1/tubulin interaction was verified by observing colocalization of the two proteins in cells and immunoprecipitation and reciprocal immunoprecipitation of tubulin and hCTPS1. Other potential hCTPS1 interacting proteins were identified by mass spectrometry from polyacrylamide gels containing hCTPS1 coimmunoprecipitating proteins. Further investigation will be needed to verify these as true hCTPS1 interacting proteins. Examination of CTPS1 expression in rat tissue demonstrated that CTPS1 was most highly expressed in the brain followed by the liver, suggesting CTPS1 may have a brain-specific function. Expression of CTPS1 in cell-lines showed that CTPS1 expression was significantly higher in HEK 293 cells than any other cell

line examined. Interestingly, differentiated 3T3 cells had decreased CTPS1 protein levels compared to undifferentiated 3T3 cells, suggesting CTPS1 may have a role in adipogenesis. Inhibition of the transcription factor c-myc is essential for the differentiation of 3T3 L1 cells and c-myc regulates expression of another pyrimidine biosynthetic enzyme, CAD, providing evidence that c-myc may regulate CTPS1 expression (Pulverer et al., 1994). Using c-myc$^{-/-}$, c-myc$^{+/+}$ and c-myc adenovirus we showed (in collaboration with Eric M. Wauson) c-myc is involved in regulating CTPS1 expression. This is the first comprehensive study of mammalian CTPS1 regulation and this data will provide a basis for future elucidation of how this critical enzyme is regulated.

5.2 Future Directions

The identification of CTPS1 as a novel GSK3 substrate points to CTPS1 as having a possible role in GSK3-mediated signaling pathways. GSK3 is involved in various signaling pathways involving insulin/IGF, Wnt, hedgehog, inflammatory cytokines and motagens (for review see (Frame and Cohen, 2001; Jope et al., 2007; Kim and Kimmel, 2000; Kim and Kimmel, 2006)) and whether CTPS1 is involved is unexplored. To investigate the role of CTPS1 in these pathways, CTPS1 activity will need to be measured and/or specifically inhibited. Preliminary experiments could be done using the CTPS inhibitor, CPEC, but to circumvent inhibition of any unintended targets of CPEC, siRNA towards CTPS1 should be employed. Reducing CTPS1 expression using siRNA also has the advantage of specifically targeting CTPS1 and not CTPS2, thereby allowing the different roles of the two isoforms in the signaling pathway being investigated to be elucidated.

In order to investigate the role of specific CTPS1 phosphorylation sites in GSK3 signaling pathways, endogenous CTPSs will need to be replaced with site-specific phosphonull mutants. A vector that coexpresses the shRNA to reduce a target of choice while simultaneously expressing a protein of interest has been developed and would be well suited for investigating the role of specific phosphorylation sites in cell signaling (Vitriol et al., 2007). To reduce the expression of endogenous CTPS1, CTPS2 or both, shRNA would need to be designed to target the 5' UTR, which will target endogenous mRNA but not mRNA transcribed from the construct encoding for the protein of interest. To determine the role of S571 phosphorylation in signaling events, expression of both CTPS isoforms will need to be reduced and S571A mutant would be expressed from this construct. Alternatively, the last thirty amino acids could be removed to form a carboxy-terminal CTPS1 truncation mutant devoid of any c-terminal phosphorylation sites (CTPS1 Δ-C-term(1-561)), which could be introduced into cells to assess whether c-terminal phosphorylation is involved in any GSK3-dependent signaling (e.g. Wnt, insulin, IGF, hedgehog, etc.).

A more physiological approach would be to generate tissue specific CTPS1 knockout mice, challenge these animals with various stimuli (e.g. insulin, IGF, etc.) and measure a physiological response such as the reduction of blood glucose levels following insulin treatment. Based on our observations of the unique regulation of CTPS1, and other enzymes and on the unique tissue localization of CTPS1 we predict that CTPS1 knockouts will have important developmental defects.

GSK3 can bind to Axin and FRAT scaffold proteins (Frequently Rearranged in Advanced T cell lymphomas), which can affect GSK3 substrate phosphorylation. To

determine whether CTPS1 is also part of the Axin or FRAT complex, CTPS1 could be immunoprecipitated and Axin or FRAT could be examined in the immunecomplex by immunoblot analysis. Alternatively and to verify any interaction observed from the CTPS1 immunoprecipitation, Axin or FRAT could be immunoprecipitated and CTPS1 could be detected by immunoblot analysis.

The significance of the Pin1 interaction is not yet clear. It is possible that this is an artifact, although our data clearly demonstrates S575 phosphorylation specific binding. One possible explanation for not observing a strong endogenous Pin1/CTPS1 interaction in HEK 293 cells is that Pin1 is mainly localized to the nucleus as where CTPS1 is mainly localized to the cytoplasm in HEK 293 cells. Since Pin1 catalyzes the cis-trans isomerization we can speculate that this interaction may influence the dynamics of phosphorylation/dephosphorylation of this protein. Thus Pin1 may provide an additional level of regulation to this complex mechanism.

The GSK3 phosphorylation site (S571) identified in CTPS1 was already mutated in a cell line from retinitis pigmentosa suggesting GSK3 and/or CTPS1 may be involved in retinitis pigmentosa. Several studies of retinitis pigmentosa have found components of the Wnt signaling pathway, which requires GSK3, aberrantly regulated (Jones et al., 2000a; Jones et al., 2000b; Pauer et al., 2005). Interestingly, a study of genetic mutations in the retinitis pigmentosa 1 protein found a threonine mutated to an isoleucine and the threonine is a consensus site for phosphorylation by GSK3 (Khaliq et al., 2005). Furthermore, inspection of the phosphorylation site database, Phosida (http://www.phosida.com), revealed two phosphorylation sites (S582 and S583) in retinitis pigmentosa-1-like protein splice isoform 1

(Q8IWN7, Swiss-Prot entry numbering). S582 is a consensus phosphorylation site for GSK3 and S583 is a consensus phosphorylation site for CK I (Olsen et al., 2006). To assess whether CTPS1 and/or GSK3 are involved in eye diseases such as retinitis pigmentosa, various mutants (e.g. CTPS1 phosphorylation site mutants or GSK3 activity mutants) can be introduced by *in vivo* electroporation and their expression controlled by 4-hydroxytamoxifen (Matsuda and Cepko, 2007). Since retinitis pigmentosa is due to the loss of photoreceptor cells by apoptosis, the death of these cells can be used to monitor the effects of the expressed protein. One possibility is that an imbalance in CTP synthesis and/or lipid synthesis may promote increased apoptosis of these cells.

The expression of CTPS1 in the brain, the evidence that GSK3 regulates CTPS1 activity and Pin1 and tubulin interact with CTPS1 suggests that CTPS1 may be involved in neurodegenerative diseases such as Alzheimer's. To investigate the potential role of CTPS1 in neurodegenerative diseases, the CTPS inhibitor, CPEC could be used with animal models that recapitulate the tauopathy observed in Alzheimer's disease and determine whether inhibition of CTPS1 decreases symptoms or the pathology associated with the disease. Additionally, nucleotide and/or phospholipid levels could be measured in animal models of Alzheimers or other neurodegenerative disorders. Expression of the mRNA of CTPS1 and CTPS2 in murine brain as determined by the Allen Institue (http://www.brainmap.org) demonstrated that both CTPSs are expressed in the cortex, cerebellum and hippocampus. The expression in the hippocampus suggests that CTPS may be involved in learning and memory. The brain region expression of CTPS1 in developing zebra fish (http://www.zfin.org) suggests that CTPS1 may be involved in brain development. To investigate the possibility that CTPS1 phosphorylation of CTPS1 is involved in brain

development, phosphorylation site mutants or CTPS1 Δ-C-term(1-561) could be introduced into developing mouse fetuses by electroporation as done previously by Dr. Franck Polleux in the department of pharmacology at the University of North Carolina at Chapel Hill. It will also be important to characterize the phosphorylation of CTPS (S571) during development. According to a model involving GSK3 regulation by Wnt and CTPS phosphorylation this site may be an important marker of CTPS regulation during this time.

In order to map the CK I phosphorylation sites in CTPS1, it would be a good idea to start by investigating if the existing phosphonull mutations of CTPS1 inhibit CK I phosphorylation *in vitro*. A panel of recombinant CK I isoforms (α, β, γ1, γ2, γ3, δ and ε) could be used to phosphorylate CTPS1 *in vitro* to determine which CK I isoform is the most efficient kinase for phosphorylating CTPS1. Preliminary results using IC261, which more specifically inhibits CK I δ and ε, suggests that these isoforms are not involved in the phosphorylation of CTPS1 (Matthew J Higgins, unpublished observations). Since CK I enzymes are considered to be constitutively active, it would be difficult to stimulate CK I and determine whether a particular potential CTPS1 phosphorylation site mutation (e.g. S571A) alters CTPS1 phosphorylation. However, CK I enzymes seem to be regulated by protein expression and localization, which could be used to target the activation or inhibition of certain CK I isoforms in intact cells. What could be done is to use isoform-specific siRNA to reduce protein expression of a particular CK I isoform and then analyze the 2D phosphopeptide maps of CTPS1 for differences. This however requires a siRNA that will inhibit expression of the CK I isoform of interest or knowledge of which CK I isoform is responsible for phosphorylating CTPS1. The best CK I inhibitor is D4476, but even this inhibitor may not be very effective in inhibiting CK I or the CK I isoform predominantly

responsible for phosphorylating CTPS1 and this will interfere with the ability to understand how CK I affects CTPS1 function. Therefore, a strategy using siRNA should be employed to overcome this problem. However, it should be noted that if multiple CK I isoforms phosphorylate CTPS1, inhibiting one isoform may not affect CTPS1 phosphorylation due to compensatory phosphorylation by another CK I isoform. Should CK I isoforms compensate for each other, a siRNA strategy (or analogous strategy) to target all CK I isoforms should be employed. Another means to identify which CK I isoform is involved in phosphorylating CTPS1 would be to immunoprecipitate CTPS1 and immunoblot for the various CK I isoforms to determine if a particular CK I isoform coimmunoprecipitates with CTPS1.

One possible mechanism for how CK I might affect CTPS1 function is by modulating the sensitivity of CTPS1 to inhibition by GSK3. To investigate this CTPS1 activity should be measured from untreated CTPS1 or CK I phosphorylated CTPS1 followed by incubation with GSK3 for various times. Should CK I modulate GSK3 inhibition of CTPS1, the prephosphorylation of CTPS1 by CK I would alter the time observed for GSK3 to alter CTPS1 activity.

Identification of the kinase responsible for phosphorylating S575 has eluded this investigation. Since S575 is followed by a proline, it is most likely phosphorylated by a proline-directed kinase, such as cyclin-dependent kinases (CDKs) or mitogen-activated protein kinases (MAPKs). The phosphorylation of CTPS1 was not altered in HeLa cells synchronized by double-thymidine block and phosphorylation of CTPS1 was not altered by the CDK inhibitors roscovitine or olomoucine, indicating that CDKs under the conditions tested were not involved in the phosphorylation of CTPS1. There is a possible cyclin binding

site in CTPS1 that could be mutated that would abrogate targeting of any CDKs to CTPS1 and thereby confirm or deny the role of CDKs in CTPS1 phosphorylation. The brain expression of CTPS1 suggests that maybe CDK5, a neuronal specific CDK, is involved in phosphorylating CTPS1. Olomoucine can inhibit CDK5 with the p35 cyclin binding partner suggesting that CDK5p35 is probably not the kinase involved, but the olomoucine study was done in HEK 293 cells where CDK5 is probably not highly expressed. Repeating this experiment in a cell type where CDK5 is expressed should be done to confirm or deny the role of CDK5 in the phosphorylation of CTPS1. Since CDK5 can use other cyclin binding partners, it is possible that CDK5 with a different cyclin binding partner is involved in phosphorylating CTPS1. Another CDK to investigate would be p34CDK4/cyclin D and p40CDK6/cyclin D3 since these CDKs were not sensitive to the CDK inhibitors used in this study (olomoucine).

Activation or inhibition of the MAPKs, JNK and ERK, did not alter CTPS1 phosphorylation indicating these kinase are not likely to be involved in CTPS1 phosphorylation. Inhibition of the other major MAPK, p38, with SB202190 did not alter CTPS1 phosphorylation (Matthew J Higgins, unpublished observation) suggesting that p38 is not involved in the phosphorylation of CTPS1 under the conditions examined.

A less biased means of identifying the kinase responsible for phosphorylating S575 would be to fractionate cell lysate on an ion-exchange column and use the fractions in an *in vitro* kinase reaction with a WT GST-c-term or S575A GST-c-term CTPS1 as substrates. Ideally, this would be done with lysate where GSK3 activity has been eliminated since phosphorylation of S571 depends on phosphorylation of S575. The proteins in the fraction

showing specificity for phosphorylating S575 could be separated by SDS PAGE (or even two-dimensional SDS PAGE) and identified by mass spectrometry. Additionally, the ion-exchange fraction containing the S575 kinase could be further purified by passing the fraction over an ATP affinity column and then subjecting the ATP affinity-bound proteins to SDS PAGE for identification by mass spectrometry. Alternatively, kinases could be identified on SDS-PAGE by performing an in-gel kinase reaction with the column fractions.

Tubulin and CTPS1 were found to be in a complex with each other. To investigate whether tubulin is a scaffold for pyrimidine biosynthesis, all the proteins proposed to be associated with tubulin should be verified by polymerizing tubulin and determining if these proteins (NDP kinase, CTPS1, cytoplasmic dynein, ribonucleotide reductase) are associated with the polymerized tubulin. Since we propose tubulin is a scaffold for some of the pyrimidine metabolizing enzymes allowing for more efficient shuttling of the product from one reaction to the next enzyme, it would be interesting if this could be recapitulated *in vitro*. One way to do this would be to isolate crude lysate (100,000 x g centrifugation), incubate [^{14}C]UDP in the presence of taxol-stimulated polymerized microtubules or unpolymerized microtubules and measure by HPLC or TLC the formation of other nucleotides (e.g CTP, CDP, dCDP, UTP, UDP).

To identify the region(s) of CTPS1 needed for the tubulin interaction, Flag tagged truncation mutants corresponding to various regions of CTPS1 (e.g. amidoligase domain, glutamine amidotransferase domain) could be constructed. To determine whether tubulin interacts with the CTPS1 truncation mutants, CTPS1 could be immunoprecipitated and the coimmunoprecipitation of tubulin could be monitored by immunoblot. Reciprocally, tubulin

could be immunoprecipitated and the Flag-tagged CTPS1 fragment be monitored via immunoblot using the α-Flag antibody. Results from coimmunoprecipitation experiments could be confirmed by immunostaining the CTPS1 fragment with α-Flag antibody and immunostaining tubulin in HEK293 cells. Fluorescent energy transfer (FRET) could also be used to monitor the tubulin/CTPS1 interaction in live cells. A CFP-CTPS1 has already been made by David Brown and HeLa cells stably expressing YFP-tubulin have already been selected for (Matthew J Higgins). CFP-CTPS1 truncation mutants could be used to determine whether the tubulin/CTPS1 interaction depends on certain regions of CTPS1 in intact cells.

An interesting possibility is that phosphorylation modulates the interaction with CTPS1 interacting proteins such as tubulin. To investigate this possibility various phosphorylation site mutants (e.g. S571I, S574A, S575A, CTPS1 Δ-C-term(1-561), etc).should be transfected and whether tubulin coimmunoprecipitation is affected by the mutant could be assessed by immunoblot. Alternatively, to determine whether CTPS1 phosphorylation affects the tubulin/CTPS1 interaction the phosphorylation of CTPS1 could be inhibited using GSK3 inhibitors and/or CK I inhibitors and the coimmunoprecipitation of tubulin monitored by immunoblot.

In addition to tubulin, several other potential CTPS1 interacting proteins were identified. To further verify that these other possible hCTPS1 interacting proteins can specifically form a complex with CTPS1, these potential CTPS1 interacting proteins should be probed for from immunoprecipitated CTPS1 using specific antibodies directed towards

the protein of interest. If an antibody does not exist for the protein of interest, then a tagged version of the protein can be expressed and the tag visualized by immunoblot.

As mentioned in chapter 3, the regulation of CTPS1 by phosphorylation may be coordinately regulated with the phosphorylation regulation of other CTP-requiring biosynthetic enzymes. The rate-limiting step in phosphatidylcholine biosynthesis, CCT, is also heavily phosphorylated on its c-terminus and phosphorylation negatively regulates this enzyme. To assess whether GSK3 can regulate phospholipid synthesis, phospholipids could be measured by TLC from cells maintained in the presence or the absence of a GSK3 inhibitor. Additionally, we would like to identify which sialylated proteins are most sensitive to CTP levels. To investigate this we will first radiolabel cells with [6-^{3}H]N-Acetyl-D-mannosamine ([^{3}H]ManNAc) an obligate precursor for sialylation of proteins, then treat cells with and without CPEC. Proteins will be separated on two dimensional gels. Gels will be Coomassie stained, dried using acetate paper and visualized by autoradiography or phosphorimager. Protein spots observed by autoradiography or phosporimager to change with CPEC treatment will be excised and identified by mass spectrometry

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