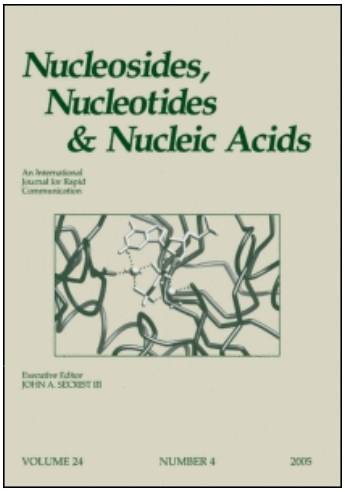

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HUMAN CYTIDINE TRIPHOSPHATE SYNTHETASE 1 INTERACTING PROTEINS

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□ *We investigated the interacting proteins and intracellular localization of CTP synthetase 1 (CTPS1) in mammalian cells. CTPS1 interacted with a GST- peptidyl prolyl isomerase, Pin1 fusion (GST-Pin1) in a Ser 575 (S575) phosphorylation-dependent manner. Immunoprecipitation experiments demonstrated that CTPS1 also bound tubulin, and thirteen additional coimmunoprecipitating proteins were identified by mass spectrometry. Immunolocalization experiments showed that tubulin and CTPS1 colocalized subcellularly. Taxol treatment enhanced this but cotreatment of cells with the CTPS inhibitor, cyclopentenyl cytosine (CPEC), and taxol failed to disrupt the colocalization. Thus, these studies provide novel information on the potential interacting proteins that may regulate CTPS1 function or intracellular localization.*

Keywords CTP; CTP synthetase; tubulin; Pin1; localization

INTRODUCTION

CTP synthetase (CTPS) is the rate-limiting step in the de novo synthesis of CTP, and we recently reported that CTPS1 was phosphorylated on multiple residues in the C-terminus.[1] However, little is known about the interacting proteins that regulate CTPS function. Based on the observation that other pyrimidine metabolic enzymes have distinct subcellular distribution (e.g., uridine phosphorylase with vimentin,[2] cytidine deaminase within the nucleus,[3]), we predicted that CTPS1 would interact with a unique subset of proteins, possibly in a phosphorylation-dependent manner. Thus the objective of this study was to identify proteins that interact with

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endogenous CTPS1 in mammalian cells primarily by immunoprecipitating CTPS1 and identifying proteins by mass spectrometry.

MATERIALS AND METHODS

Glutathione-S-Transferase (GST) and *Xenopus laevis* GST-Pin1 (GST-xPin1) were kind gifts from Dr. Anthony Means (Duke University). Cyclopentenyl cytosine (CPEC) was obtained from the National Cancer Institute.

Transfection of HEK293 Cells and Pin1 RNA Interference

Pin1 immunoblotting was performed as described by the manufacturer (Upstate Biotechnology, Lake Placid, NY, USA). Human embryonic kidney (HEK) 293 cells (American Tissue Culture Company, ATCC, Rockville, MD, USA) were maintained in DMEM(Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Transfection of HEK293 cells with human CTPS1 and silencing of Pin1 was done as described for the silencing of GSK3β using SmartPool siRNA targeting Pin1 from Dharmacon (Lafayette, CO, USA; catalog number 003291).[1]

Immunostaining

Cells were fixed as described previously,[4] α-CTPS1 (1:100) and α-tubulin (1:500, DM1A clone from Sigma) diluted in blocking solution were incubated overnight with coverslips. Following washing, secondary antibodies conjugated either to Alexa 488 or Alexa 594 (Invitrogen) fluorophore were added to coverslips and incubated at room temperature in the dark for 1 hour. After washing, coverslips were mounted onto glass slides with Fluorosave (Calbiochem, San Diego, CA, USA).

Protein Identification

After separation of proteins by SDS-PAGE, proteins were trypsinized and the tryptic peptides were used to identify proteins by peptide mass fingerprinting on a 4700 matrix assisted laser desorption ionization (MALDI) time of flight/time of flight (TOF/TOF) mass spectrometer (Applied Biosystems, Foster City, CA, USA). Peptides masses were searched against the Swiss protein database using the Mascot search engine.[5] Proteins containing protein scores above 90 were listed.

RESULTS

Pin1 Interacts with CTPS1

Pin1 is a peptidyl prolyl isomerase that recognizes phosphorylated serines or threonines immediately N-terminal to prolines and catalyzes the cis to trans isomerization of the prolyl peptide bond.[6,7] We recently demonstrated that CTPS1 was phosphorylated by GSK-3 and since some of the Pin1 targets are known substrates of GSK-3 (e.g., tau protein), we investigated whether phosphorylated CTPS1 might also be a Pin1 target.[1] Coimmunoprecipitation experiments demonstrated that GST-xPin1 and CTPS1 formed a complex whereas little interaction was observed with GST alone. (Figure 1A).To determine if this interaction was dependent on S575 phosphorylation, GST-xPin1 was incubated with lysate from untransfected or HEK293 cells transfected with wild type (WT) or S575A flag-tagged mutant. The CTPS1 S575A mutant protein showed decreased ability to interact with GST-xPin1 compared to WT-CTPS1 demonstrating that Pin1 binding was S575 phosphorylation dependent (Figure 1A). To determine whether Pin1 binding could alter CTPS1 activity, Pin1 expression was reduced by transfection of HEK293 cells with SmartPool siRNAs directed towards Pin1 and CTPS1 activity determined. Although siRNA treatment greatly reduced Pin1 expression, CTPS1 activity was not affected, indicating that under these conditions loss of Pin1 did not alter the activity or expression of CTPS1 (Figure 1B).

Screen for Other CTPS1 Interacting Proteins

The following strategies were used to identify other CTPS1 interacting proteins: 1) CTPS1 was immunoprecipitated from HEK293 cells using the CTPS1 antibody preabsorbed with or without the antigenic peptide; 2) coimmunoprecipitating proteins were separated by SDS PAGE; 3) cells were radiolabeled with [^{35}S]-methionine (ICN, Irvine, CA, USA) to enhance protein detection; 4) interacting proteins were identified by MALDI TOF mass spectrometry. Examination of proteins from [^{35}S]-methionine labeled cells showed that in addition to CTPS1, tubulin (peptides identifying both α and β tubulin) was identified as an interacting protein. Additionally, deoxyhupusine synthase (DHS) was also identified as an interacting protein by this method (Figure 1C).

Immunoprecipitation and Reciprocal Immunoprecipitation of CTPS1 and Tubulin

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,

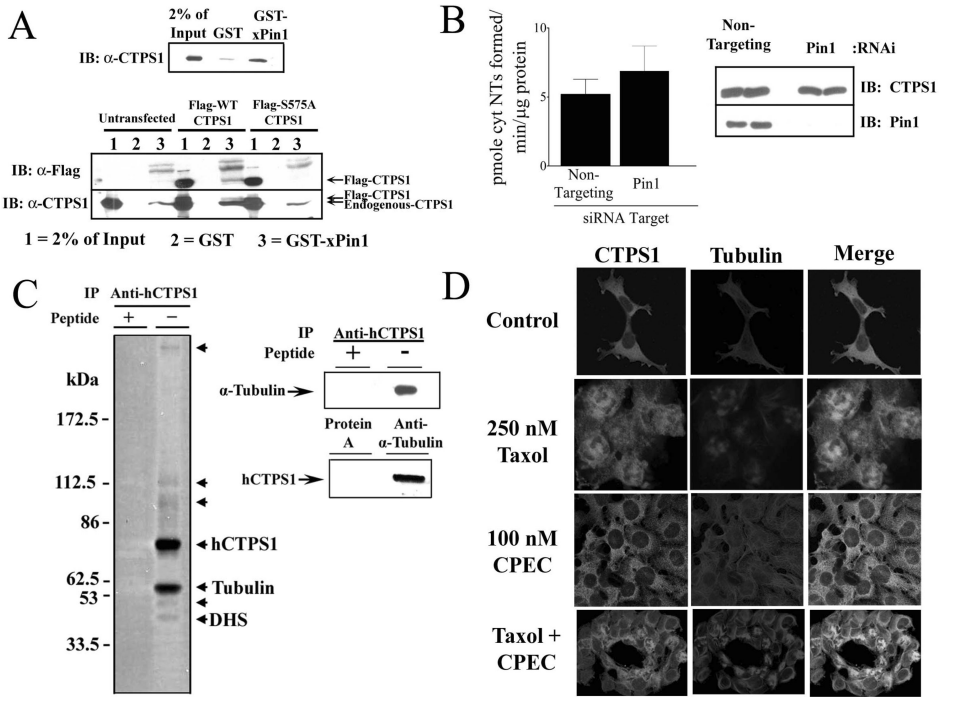

FIGURE 1 Pin1 and Tubulin interaction with CTPS1. A) HEK 293 cell lysates were incubated with approximately 20 μg of GST or GST-xPin1. Transfected CTPS1 was visualized by α-Flag immunoblot (IB) and endogenous CTPS1 was visualized using an antibody specific for CTPS1 as described previously.[1] 2% input refers to 2% of the total amount of protein used in the immunoprecipitation. B) 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 Higgins et al.[1] Expression of Pin1 (α-Pin1, Upstate Biotechnologies) and CTPS1 was assessed by immunoblot. C) HEK293 cells were labeled overnight with [^{35}S]-methionine and immunoprecipitated 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. Anti-CTPS1 antibody or anti-CTPS1 antibody preabsorbed with antigenic peptide was used to immunoprecipitate CTPS1 from HEK 293 cells. Coimmunoprecipitating proteins were separated by SDS PAGE and α-tubulin (α-alpha tubulin (DM1A), Sigma) was visualized by immunoblot analysis. Anti-α-tubulin antibody or protein A alone was used to immunoprecipitate α-tubulin from HEK 293 cell lysates as described in Materials and Methods. CTPS1 was visualized by immunoblotting for CTPS1. D) Immunostaining of CTPS1 and tubulin was done as described in materials and methods using Alexa 488 conjugated secondary for staining of CTPS1 and Alexa 594 conjugated secondary for staining anti-tubulin. HEK 293 cells were treated with 100 nM taxol for 7 hours prior to fixing cells. HEK 293 cells were treated with 100 nM CPEC for 48 hours prior to fixing cells. HEK 293 cells were treated with 100 nM CPEC and 250 nM taxol for 7 hours prior to fixing cells for immunostaining.

indicating that this interaction was CTPS1-dependent (Figure 1C). 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 1C).

Colocalization of Tubulin with CTPS1 in HEK 293 Cells

To examine the colocalization of CTPS1 with microtubules in intact cells, HEK293 cells were treated with the microtubule stabilizing agent, taxol, and the samples immunostained for CTPS1 and microtubules. As seen in Figure 1D, taxol induced the formation of "tufts" of microtubules and CTPS1 colocalized with these tufts, indicating that under conditions where microtubule depolymerization was inhibited, the colocalization of CTPS1 and microtubules was enhanced.

To determine if inhibition of CTPS affected microtubule association, HEK 293 cells were treated with cyclopentenyl cytosine (CPEC), a highly specific inhibitor of CTPS. Treatment with CPEC appeared to increase the size of cell nuclei and the network of microtubules (Figure 1D). In cells where "tufts" were not observed, the microtubule network seemed to be surrounding the enlarged nucleus (Figure 1D). CTPS1 colocalized with "tufts" of microtubules in the presence of CPEC and taxol and appeared to colocalize with the microtubule network surrounding the nucleus in cells where the "tufts" were absent (Figure 1D). We also investigated whether cotreatment with CPEC and taxol altered the microtubule architecture and/or CTPS1 colocalization. The cotreatment of HEK 293 cells with CPEC plus taxol induced the "tufts" of microtubules to form. The fact that CPEC did not prevent colocalization of CTPS1 with taxol-induced microtubule "tufts" indicated that CTPS activity may not be required for this colocalization.

Identification of Other CTPS1-Interacting Proteins

Further investigation using the same strategy as above and colloidal Coomassie staining of the SDS PAGE gel, revealed thirteen coimmunoprecipitating proteins (Figure 2). Included was the multi-enzyme carbamoyl phosphate synthetase, aspartate transcarbamylase, dihydrooratase (CAD) required for de novo synthesis of pyrimidines, and multiple cytoskeletal proteins.

DISCUSSION

The results of this study identified Pin1 as a novel phosphorylation-dependent CTPS1 interacting protein. Although reduction of Pin1 expression did not affect CTPS1 activity under our experimental conditions, Pin1 may still regulate CTPS1 activity or localization in intact cells. These studies also identified tubulin as a CTPS1 interacting protein. This interaction appears to be direct and is supported by immunostaining of CTPS1 and tubulin in intact cells. Tubulin may act as a scaffold protein for pyrimidine biosynthetic enzymes allowing products from one reaction to be efficiently channeled to the next enzyme. For example, nucleoside diphosphate

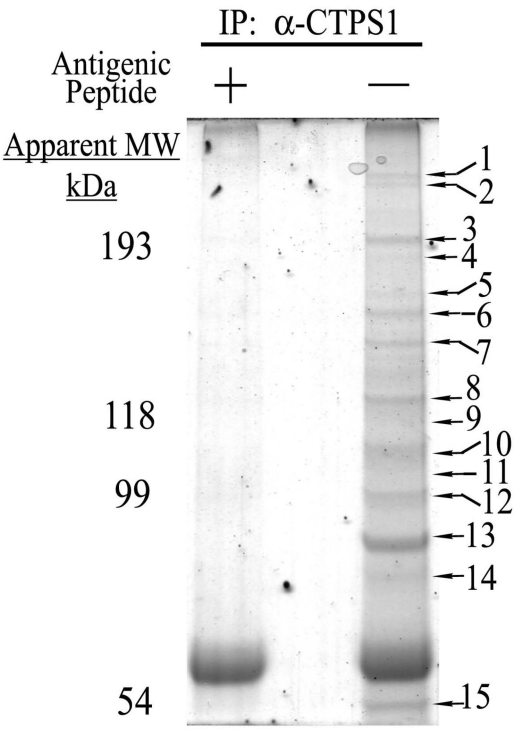

FIGURE 2 Screen of CTPS1 interacting proteins. CTPS1 was immunoprecipitated with anti-CTPS1 antibody or anti-CTPS1 antibody preabsorbed with antigenic peptide. Indicated coomassie stained protein bands were excised, trypsin digested and identified by peptide mass fingerprinting on a ABI 4700 MALDI TOF/TOF.

(NDP) kinase interacts with tubulin[8] and forms UTP and GTP, two nucleoside triphosphates important in the CTPS1 reaction. Thus polymerized microtubules may facilitate more efficient shuttling of NDP products, UTP and GTP, into the CTPS reaction.

Other interacting proteins discovered in this screen include cytoplasmic dynein, deoxyhypusine synthase and CAD (Table 1). Whether or not these are direct interactions with CTPS1 or indirect interactions through binding tubulin or other intermediary proteins remains to be determined.[9,10] Interestingly, cytoplasmic dynein can hydrolyze CTP to CDP, a substrate for ribonucleotide reductase, further supporting the notion that tubulin may act as a scaffold for pyrimidine biosynthetic enzymes. CAD, a pyrimidine biosynthetic enzyme, was previously identified as a CTPS interacting protein in a large scale analysis of the yeast interactome.[11] Thus, it is interesting to speculate that a complex of enzymes involved in the synthesis or metabolism of pyrimidines may be organized through interactions with tubulin or each other.

TABLE 1 List of proteins identified to co-immunoprecipitate with CTPS1. Given is the NCBI accession number

Band Number	Protein Name	Accession Number	Protein Score
1	Cytoplasmic dynein heavy chain	Q14204	399
2	DNA dependent protein kinase catalytic subunit	P78527	278
3	Filamin A	P21333	159
4	Fatty Acid Synthase	P49327	247
	GCN (general control of amino-acid synthesis)-like protein	Q92616	150
	CAD (glutamine-dependent carbamoyl-phosphate synthase; aspartate carbamoyltransferase; dihydroorotase)	P27708	128
5	Pericentrin	O95613	96
	IQGAP (Ras GTPase-activating-like protein) p195	P46940	91
6	Bifunctional t-RNA synthetase glutamyl	P07814	155
	hemoglobin-haptoglobin binding protein A	Q48153	115
7	Isoleucyl t-RNA synthetase	P41252	117
	Spectrin	P13395	101
8	Spectrin	Q00963	101
9	Not Identified		
10	Spectrin	Q9NRC6	113
	Golgin subfamily A member 4	Q13439	101
11	Microtubule/actin cross-linking factor	QUPN3	127
12	Nucleopore complex protein Nup93	Q8N1F7	94
	HSP (heat shock protein) 90	P08238	94
13	CTP synthetase 1	P17812	140
14	Not identified		
15	Retinitis pigmentosa (mouse)	Q8CGM2	134
	Microtubule/actin cross-linking factor	Q96PK2	109

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