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EXTRA VIEW

Ubiquitination and filamentous structure of cytidine triphosphate synthase

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ABSTRACT

Living organisms respond to nutrient availability by regulating the activity of metabolic enzymes. Therefore, the reversible post-translational modification of an enzyme is a common regulatory mechanism for energy conservation. Recently, cytidine-5′-triphosphate (CTP) synthase was discovered to form a filamentous structure that is evolutionarily conserved from flies to humans. Interestingly, induction of the formation of CTP synthase filament is responsive to starvation or glutamine depletion. However, the biological roles of this structure remain elusive. We have recently shown that ubiquitination regulates CTP synthase activity by promoting filament formation in *Drosophila* ovaries during endocycles. Intriguingly, although the ubiquitination process was required for filament formation induced by glutamine depletion, CTP synthase ubiquitination was found to be inversely correlated with filament formation in *Drosophila* and human cell lines. In this article, we discuss the putative dual roles of ubiquitination, as well as its physiological implications, in the regulation of CTP synthase structure.

Introduction

Allosteric regulation and reversible post-translational modification of metabolic enzymes are common mechanisms to achieve a rapid response to environmental changes and energy conservation. Many reversible covalent modifications of metabolic enzymes critically regulate enzymatic activities, such as phosphorylation, nucleotidylation, ADP-ribosylation, acetylation, methylation, and ubiquitination.[1] Accumulating evidence indicates that the polymeric assembly of metabolic enzymes and the reversible association of sequential enzymes in a metabolic pathway could also serve as important regulatory mechanisms for enzymatic activities.[1] In our recent report, we linked ubiquitination to regulation of the polymeric structure of cytidine triphosphate synthase (CTPsyn), which is known as the cytoophidium in flies, and revealed a role of the cytoophidia in CTP synthesis for maintaining the nucleotide pool during endoreplication.[2] Our results suggest that the ubiquitination-mediated polymerization of CTPsyn

might serve as a regulatory mechanism for enzymatic activity. Here, we discuss the possibility that this ubiquitin-related regulation is a conserved mechanism in *Drosophila* and humans, and further elucidate the potential molecular mechanism of ubiquitin modification on the assembly of CTPsyn filaments.

Enzymatic activity and the polymeric structure of CTPsyn

The nucleotide CTP is a basic building block for DNA, RNA, and phospholipids synthesis.[3] CTPsyn functions as the rate-limiting step in the *de novo* synthesis of CTP, and catalyzes the formation of CTP from uridine 5′-triphosphate (UTP) in an adenosine 5′-triphosphate (ATP)-dependent reaction, using either ammonia or glutamine as a nitrogen source in bacteria or eukaryotes, respectively.[4-10] CTPsyn has 2 major domains, a C-terminal glutamine-dependent aminotransferase (GATase)/glutaminase domain and an N-terminal synthetase domain.[11-12] The GAT domain

binds to glutamine and releases ammonia to the synthetase domain which binds to the substrate UTP and promotes its activation by reacting with ATP,[11-12] with the subsequent replacement of the phosphate group of UTP with ammonia. Biochemical studies have shown that CTPsyn exists as an inactive monomer at lower protein concentrations, but that the enzymes polymerize to form a dimer at increased enzyme concentrations, and this inactive dimer forms an active tetramer in the presence of ATP and UTP.[5,13] Moreover, guanosine 5′-triphosphate (GTP) allosterically enhances enzymatic activity by altering the 3-dimensional structure of the CTPsyn tetramer.[14-15] CTP also plays an inhibitory feedback role by competing with UTP binding. Taken together, CTPsyn plays a central role in monitoring the balance of nucleotide pools.[16]

CTPsyn has been shown to form a highly conserved filamentous structure in various organisms, including bacteria, yeast, *Drosophila* and mammalian cell lines.[17-20] However, the importance of the polymeric structure of CTPsyn for regulating enzyme activity remains unclear. In a study with *E. coli*, CTPsyn filaments were disassembled in the presence of substrates (UTP and ATP), which resulted in an immediate increase in CTP production. On the other hand, CTPsyn filament formation was induced by the addition of the end-product CTP.[21] Moreover, mutations at the tetramer stacking interface that cause filament formation failures also led to higher enzymatic activity.[21] These results suggest that CTPsyn in a filamentous form might be less active, and end product feedback inhibition could trap CTPsyn in the filament structures.[21] In yeast, CTPsyn filaments can be regulated by changes in nutrient status, in which saturated culture and glucose depletion were shown to induce CTPsyn filament formation.[19] Mutations in the substrate binding site (for ATP/UTP) and the allosteric regulator binding site (for GTP) exhibited a higher frequency of filament formation compared with the wild type.[22] These data suggest that CTPsyn filaments are composed of inactive CTP synthase.

In the fly, CTPsyn filaments have been directly observed in various tissues, such as in the ovary, and were found to be induced by starvation in other tissues such as imaginal discs.[17,23] Two different sizes of filaments were observed in the ovary: cytoophidia, which are mainly located in follicle cells, and macro-cytoophidia, which mainly exist in nurse cells and the oocyte. The macro-cytoophidia were shown to be regulated by the co-localized *Drosophila* activated cdc42-associated kinase (DAck) protein and were found to be composed of catalytically active CTPsyn, suggesting that filament formation facilitates or permits enzyme activity.[24] Consistent with our recent study,[2] this suggests that the cytophidia in follicle cells provide CTPsyn with its enzymatic activity during endocycles. The CTPsyn filament disassembled in the absence of casitas B-lineage lymphoma (Cbl), leading to a defect in the S phase, and this defect was restored by overexpression of a wild-type CTPsyn, but not an enzymatically inactive, mutant CTPsyn transgene.[2] This provides a link between the CTPsyn filament and enzymatic activity. In the *Drosophila* S2 cell culture system, under normal culture conditions, only 5% of the cells contained visible CTPsyn filaments (Fig. 1A) which is similar to the frequency reported for previously studied human cell lines.[20] However, in S2 cells CTPsyn filaments were found as dimeric forms of CTPsyn-GFP, suggesting that the filaments could down-regulate enzymatic activity.[23] In mammals, although some primary cells (e.g., pluripotent embryonic stem cells) spontaneously form filaments, filament formation could also be induced by glutamine deprivation in most cancer cell lines.[25,26] In addition, glutamine analogs, such as 6-diazo-5-oxo-L-norleucine (DON) or Acivicin, could cause irreversible assembly of CTPsyn into filaments.[2,20,27] Taken together, it appears that CTPsyn filament formation could be induced by limited nutrition as well as by feedback inhibition from end-product binding. Under both conditions, CTPsyn filaments may be composed of inactive enzymes. However, naturally existing CTPsyn filaments might be different, since there is generally no nutrition limitation or excessive CTP. Indeed, both the macro-cytoophidia in the germline cells and the cytoophidia in follicle cells seem to provide CTPsyn with its enzymatic activity for phospholipids and DNA synthesis.[2,24] Furthermore, we found that inactive exogenous CTPsyn (CTPsyn^{C399G}) could be incorporated with endogenous wild-type CTPsyn to form filaments in follicle cells.[2] Therefore, it is likely that the CTPsyn filaments are heterogeneous, and should not be recognized as a single entity based simply on morphological similarities.
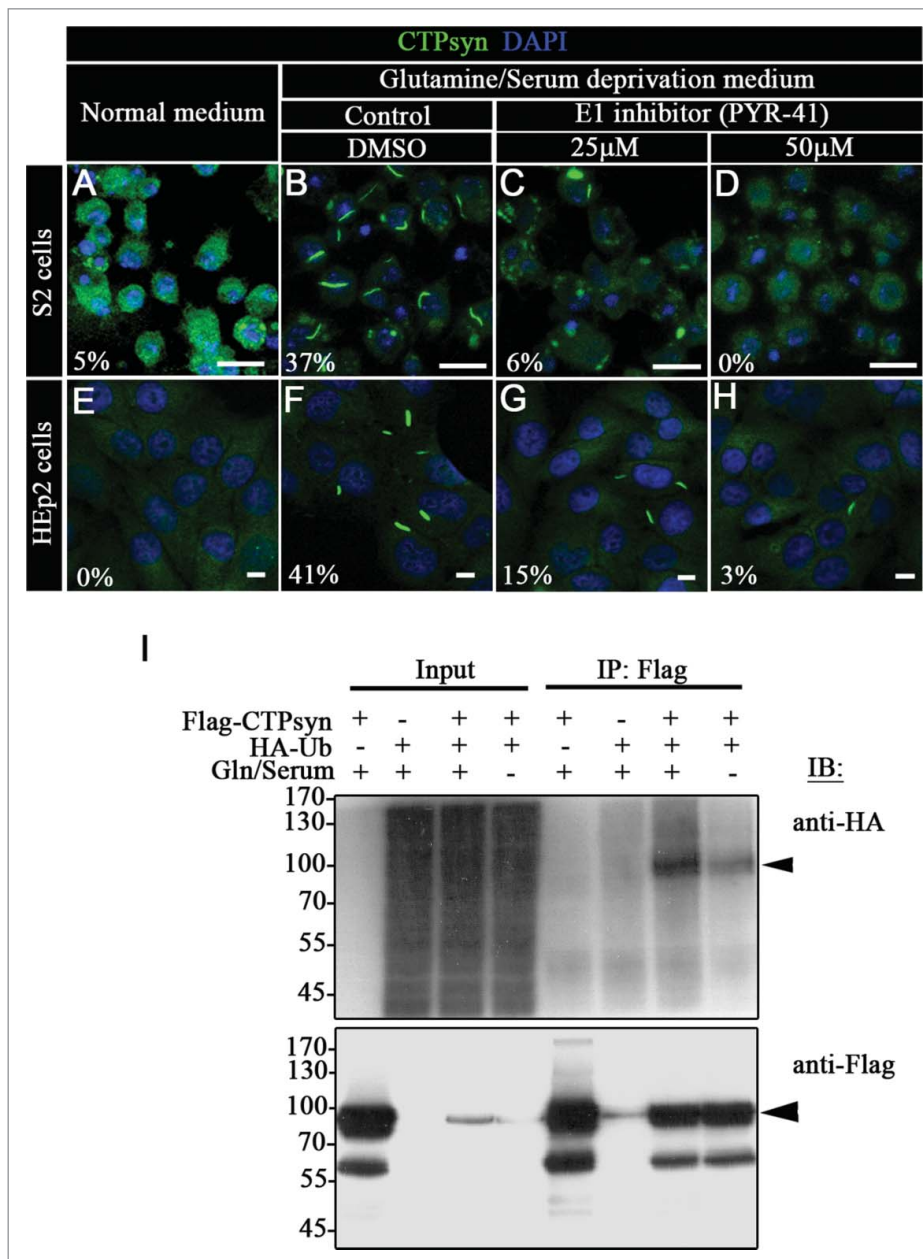
Figure 1. Ubiquitination is required for CTPsyn filament formation in both *Drosophila* and mammalian cells. S2 cells (A–D) and HEp2 cells (E–H) incubated in glutamine/serum-deficient medium without (B and F) or with 25 μM (C and G) or 50 μM (D and H) of the E1 inhibitor (PYR-41); cells were stained with anti-CTPsyn (green) and DAPI (blue). The percentages indicate the proportions of CTPsyn filaments among total cells. The CTPsyn filament structures were significantly reduced from 37% (B) to 6% (C), and 0% (D) of S2 cells with 25 μM and 50 μM PYR-41 treatment, respectively. The percentages of HEp2 cells with CTPsyn-filaments were markedly reduced from 41% (F) to 15% (G) and 3% (H) with 25 μM and 50 μM PYR-41 treatment, respectively. (I) S2 cells were transfected with HA-Ub and Flag-CTPsyn and incubated in complete or glutamine/serum-deficient Schneider's medium. The CTPsyn-filamentous complexes were immunoprecipitated with anti-Flag and were then probed with anti-HA (arrowhead in upper panel) and anti-Flag antibodies (arrowhead in lower panel). Scale bar: 10 μm.

Ubiquitination-mediated regulation of CTPsyn filaments in *Drosophila* and humans

Post-translational modification of metabolic enzymes is a regulation strategy for rapidly responding to environmental changes. A well-known example of such modification is acetyl-CoA carboxylase which forms a polymeric filamentous structure through signaling-mediated protein dephosphorylation.[28,29] Interestingly, this polymer form of acetyl-CoA carboxylase is enzymatically active. CTPsyn has been reported to be phosphorylated at various serine and threonine residues by protein kinase C, protein kinase A and

glycogen synthase kinase 3, which in turn up-regulate or downregulate the catalytic activity of CTPsyn.[30-33] However, studies in *Saccharomyces cerevisiae* and *Drosophila* indicated that phosphorylation of CTPsyn is not a key regulatory mechanism in filament formation.[24,34]

We recently demonstrated that an ubiquitin-modification system is involved in the maintenance of CTPsyn filaments in the *Drosophila* ovary and salivary glands.[2] Depletion of ubiquitin with the proteasome inhibitor MG132 disrupted CTPsyn filaments in follicle cells, and reduced the length of the macro-cytoophidia in the nurse cells and oocyte of *Drosophila* ovary.[2] These defects could be rescued by overexpression of ubiquitin, or treatment with a deubiquitinase inhibitor PR-619. This work further identified Cbl as an E3 ligase that stabilizes the CTPsyn filament during the endocycle. Loss of Cbl function results in disruption of CTPsyn filaments, with subsequent reduction of S-phase events during the endocycle. However, the effects of Cbl were observed only in follicle cells at the endocycle stages, but not in germline cells or follicle cells at the mitotic stages.[2] These results suggest that ubiquitination may play a universal role in the regulation of CTPsyn filament formation, and that Cbl is a specific regulator of follicle cells in endocycles. The polyubiquitination or multi-monoubiquitination modification of proteins could serve to controls protein stability or protein-protein interactions, respectively.[35] Since the protein levels of CTPsyn were not altered in a *Cbl* mutant, the ubiquitination required for filament formation is more likely related to protein-protein interactions.[2]

To determine whether ubiquitination represents an evolutionarily conserved regulatory mechanism, the role of ubiquitination in CTPsyn filament formation was further examined in *Drosophila* S2 and human HEp2 cells, a human laryngeal carcinoma cell line. Very few S2 and HEp2 cells contain CTPsyn filaments under normal culture conditions (Fig. 1A and 1E). However, under glutamine/serum depletion, approximately 35% and 40% of S2 and HEp2 cells, respectively, formed filaments (Fig. 1B and 1F). Interference with the ubiquitination pathway in S2 cells with 25 μM and 50 μM PYR-41, an E1 (ubiquitin-activating enzyme) inhibitor, significantly reduced CTPsyn filament formation in a dose-dependent manner (Fig. 1C–1D). A similar phenomenon was observed in HEp2 cells, with a marked reduction in the number of CTPsyn filament-positive cells following treatment of PYR-41 (Fig. 1G–1H). These results indicate that ubiquitination is required for nutrient-depletion induced CTPsyn filaments, and this requirement also holds for naturally existing CTPsyn filaments in follicle cells and germline cells.

The ubiquitination of CTPsyn is inversely correlated with CTPsyn filament formation in Drosophila S2 cell

To determine whether ubiquitination occurs on the protein of CTPsyn, the ubiquitin modification of CTPsyn was directly examined by overexpression of Flag-CTPsyn and HA-tag ubiquitin in S2 cells, followed by immunoprecipitation with anti-Flag antibodies. The ubiquitinated-CTPsyn could be readily observed in S2 cells when cultured with complete Schneider's medium, but the proportion of ubiquitinated-CTPsyn was gradually reduced in glutamine/serum-depleted medium (Fig. 1I). These results indicate that some degree of ubiquitination detected on soluble form of CTPsyn was reduced when CTPsyn formed higher-order structures, such as filaments (Fig. 1). This result suggests that the ubiquitin modification of CTPsyn is negatively associated with CTPsyn filament formation. Furthermore, the immunoprecipitation results showed that the ubiquitin modification of CTPsyn is most likely to be in the form of monoubiquitination, based on the molecular weight change in SDS-PAGE (Fig 1I). We hypothesize that monoubiquitination may modulate protein-protein interaction instead of degradation. Although the ubiquitination of CTPsyn was also reported in a proteomic analysis,[36] it is not yet clear whether this modification is crucial for CTPsyn activity, and the specific residues that are modified have yet to be determined. Identification of the residues involved in the ubiquitin modification of CTPsyn may reveal the physiological effect of this modification, such as substrate access due to conformational alternation.

Conclusion

In summary, our results indicate that ubiquitin modification plays a crucial role in the regulatory mechanism of CTPsyn filament formation and is conserved from flies to mammals. In the *Drosophila* ovary, where filaments exist naturally, this structure leads to efficient CTP production for rapid phospholipid and DNA synthesis.[2,24] In addition, studies in bacteria and *Drosophila* indicate that the newly synthesized CTPsyn becomes incorporated into the filament structure in a cooperative manner.[2,21]
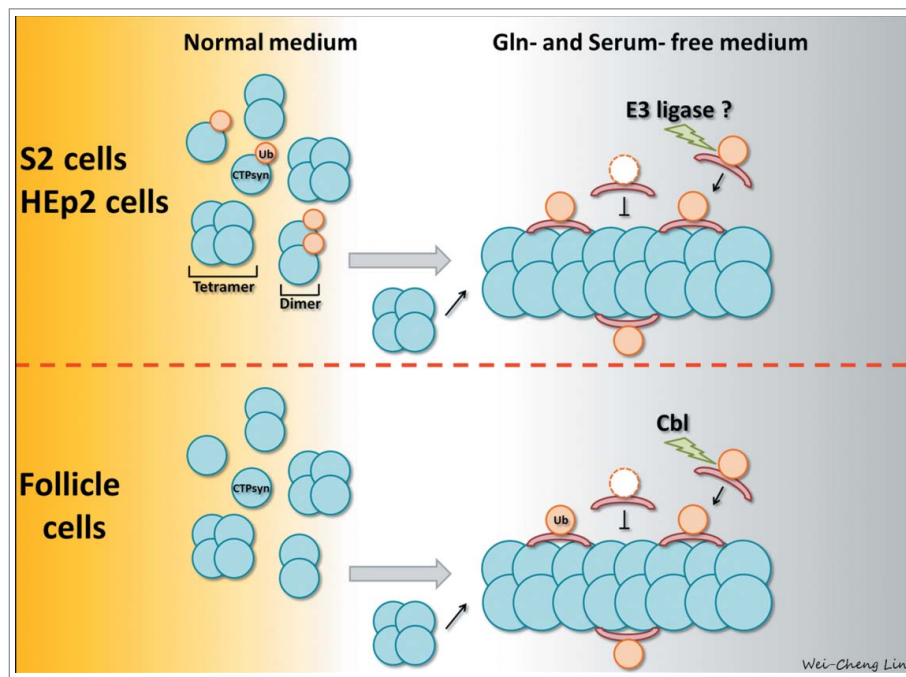
Figure 2. A proposed model for the role of ubiquitination in CTPsyn filament assembly. In normal conditions, CTPsyn can exist as an inactive monomer or dimer, or as active/inactive tetramers. Ubiquitination of CTPsyn appears to be unrelated to filament assembly; instead, the ubiquitination of some unknown protein might be crucial for the compartmentalization of CTPsyn into filamentary structures.

Therefore, we propose that the CTPsyn-filament structure may serve as a reservoir for rapid activation. Interestingly, the ubiquitinated form of CTPsyn was not observed in the ovary or salivary gland, where CTPsyn filaments naturally exist.[2] Taken together, our data suggest that CTPsyn filament formation may depend on ubiquitin modification of a yet-to-be components in the CTPsyn filament complex or in other molecules in a regulatory pathway (Fig. 2). To identify this crucial regulator, further analyses of CTPsyn filament components and regulatory pathways are essential. Taken together, ubiquitination likely regulates the polymeric structure of CTPsyn in distinct ways. One modification is on the CTPsyn enzyme itself, which might be removed prior to filament assembly, whereas the other is on the CTPsyn complex- associated proteins, which might be required for filament maintenance. Further experiments are required to elucidate the specific effects of these modifications.

Abbreviations and acronyms

CTP	Cytidine-5′-triphosphate
ATP	Adenosine-5′-triphosphate
UTP	Uridine-5′-triphosphate
GTP	Guanosine-5′-triphosphate
ADP	Adenosine diphosphate
DON	6-Diazo-5-oxo-L-norleucine
DAck	*Drosophila* activated cdc42-associated kinase
GATase	Aminotransferase domain
Cbl	Casitas B-linage lymphoma
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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