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Human holocarboxylase synthetase with a start site at methionine-58 is the predominant nuclear variant of this protein and has catalytic activity

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Abstract

Holocarboxylase synthetase (HLCS) catalyzes the covalent binding of biotin to both carboxylases in extranuclear structures and histones in cell nuclei, thereby mediating important roles in intermediary metabolism, gene regulation, and genome stability. HLCS has three putative translational start sites (methionine-1, -7, and -58), but lacks a strong nuclear localization sequence that would explain its participation in epigenetic events in the cell nucleus. Recent evidence suggests that small quantities of HLCS with a start site in methionine-58 (HLCS58) might be able to enter the nuclear compartment. We generated the following novel insights into HLCS biology. First, we generated a novel HLCS fusion protein vector to demonstrate that methionine-58 is a functional translation start site in human cells. Second, we used confocal microscopy and western blots to demonstrate that HLCS58 enters the cell nucleus in meaningful quantities, and that full-length HLCS localizes predominantly in the cytoplasm but may also enter the nucleus. Third, we produced recombinant HLCS58 to demonstrate its biological activity toward catalyzing the biotinylation of both carboxylases and histones. Collectively, these observations are consistent with roles of HLCS58 and full-length HLCS in nuclear events. We conclude this report by proposing a novel role for HLCS in epigenetic events, mediated by physical interactions between HLCS and other chromatin proteins as part of a larger multiprotein complex that mediates gene repression.

Keywords

biotin; carboxylases; holocarboxylase synthetase; nucleus; translation

INTRODUCTION

Holocarboxylase synthetase (HLCS) plays a pivotal role in biotin-dependent metabolic and epigenetic phenomena in humans. In intermediary metabolism, HLCS catalyzes the covalent

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binding of biotin to five distinct carboxylases [1; 2; 3]. Biotinylated carboxylases are key enzymes in the metabolism of glucose, fatty acids, and leucine [4]. In the regulation of genes by epigenetic phenomena, HLCS translocates to the cell nucleus [5] where it binds to chromatin [6; 7] to catalyze the binding of biotin to histones H1, H3, H4 and, to a lesser extent, H2A [8; 9; 10; 11; 12; 13]. Moreover, evidence suggests that HLCS interacts physically with the methylated cytosine binding protein MeCP2 and the histone H3 K9-methyl transferase EHMT-1 [14; Yong et al., unpublished].

Biotinylated histones are enriched in transcriptionally repressed loci and repeat sequences [15; 16]. Importantly, evidence suggests a role for K12-biotinylated histone H4 and HLCS in the transcriptional repression of retrotransposons, and that low abundance of K12-biotinylated histone H4 in HLCS- or biotin-deficient cells is linked with activation of retrotransposons and chromosomal abnormalities [17]. It is currently uncertain whether the effects of HLCS in epigenetic pathways are mediated by HLCS-dependent biotinylation of histones or by physical interactions of HLCS with other chromatin proteins such as MeCP2 and EHMT-1. If the latter proved correct, then biotinylation of histones would be a mark to trace loci where HLCS interacts with other chromatin proteins.

Consistent with the important roles of HLCS in intermediary metabolism and epigenetics, no living HLCS null individual has ever been reported, suggesting embryonic lethality. HLCS knockdown studies (~30% residual activity) produced phenotypes such as decreased life span and heat resistance in *Drosophila melanogaster* [6] and aberrant gene regulation in human cell lines [16; 17; 18]. Mutations in the human *HLCS* gene cause a substantial decrease in HLCS activity and metabolic abnormalities [19; 20]. Unless diagnosed and treated early, HLCS deficiency is uniformly fatal [21].

Human *HLCS* is a single copy gene, which maps to chromosome 21q22.1 [1] and codes for a full-length protein of 726 amino acid with a predicted molecular weight of 81 kDa [22]. Three HLCS transcripts plus additional splicing variants originate in exons 1, 2, and 3 of the gene [22]; methionines-1, -7, and -58 in exons 6 and 7 have been identified as possible translation start sites [23]. HLCS proteins, migrating with apparent sizes of 62, 64, 76, 82, and 86 kDa in gel electrophoresis, have been detected in human placenta and bovine liver by using anti-HLCS antibodies, but some of the bands might have been caused by protein degradation [23; 24]. Presumably, the 76-kDa, 82 kDa, and 86 kDa bands represent HLCS with translation start sites in methionine-58, methionine-7, and methionine-1, respectively.

A recent study suggests that HLCS with a translation start site in methionine-58 (HLCS58) might enter the cell nucleus [25]. In this study we sought to determine (i) whether methionine-58 in HLCS is a functional translation start site; (ii) whether HLCS58 enters the cell nucleus; and (iii) whether HLCS58 has biotin protein ligase activity *in vitro*. Human embryonic kidney HEK-293 cells were chosen as model in cell culture studies to facilitate comparisons with a recent report on HLCS distribution in human cells [25].

MATERIALS AND METHODS

Plasmids

HLCS was fused to the N-terminus of enhanced green fluorescent protein (GFP) to allow for tracking the subcellular distribution of HLCS. Briefly, full-length HLCS (amino acids 1 – 726) was PCR amplified using primers 5'-GGGACTCGAGATGGAAGATAGACTCCACATGG-3' (forward) and 5'-ATTTGAATTCGCCGCCGTTTGGGGAG-3' (reverse). The PCR product was fused to the N-terminus of plasmid pEGFP-N1 (Clontech, Mountain View, CA, USA) by using *XhoI* and *EcoRI*. The plasmid was denoted HLCS-GFP and codes for a fusion protein of ~110 kDa. A

fusion protein lacking the 57 N-terminal amino acids of HLCS was created by substituting the forward primer 5'-GGGACTCGAGATGGAGCATGTTGGC-3' for the forward primer used for full-length HLCS. The plasmid was denoted HLCS58-GFP and codes for a fusion protein of ~103. kDa. Finally, a fusion protein was created in which methionine-58 was mutated to leucine-58 to prevent translation at position 58. First, DNA coding for amino acids 59 – 722 in HLCS was PCR amplified using the primers 5'-CGGAAGCTTGAGCATGTTGGCAGAGATG-3' (forward) and 5'-ATTTGAATTGCGCCGCGTTTGGGGAG-3' (reverse); the PCR product was digested with *HindIII* and *EcoRI*, and cloned into vector pEGFP-N1 to create HLCS59-GFP. Second, DNA coding for amino acids 1 – 57 in HLCS was amplified using the primers 5'-TTCTCGAGATGGAAGATAGACTCCACATGG-3' (forward) and 5'-GGAAGCTTACCGTCCTGCTCAGG-3' (reverse); the PCR product was digested with *XhoI* and *HindIII*, and cloned into plasmid HLCS59-GFP to create a fusion construct coding for full-length HLCS and GFP with the Met58Leu mutation (denoted HLCS58m-GFP; ~110 kDa). All constructs were verified by DNA sequencing of the entire protein-coding region.

Transformation of HEK-293 cells

HEK-293 cells were cultured in Dulbecco's modified Eagle's medium using routine procedures [26]. Cells were transfected with HLCS-GFP, HLCS58-GFP, and HLCS58m-GFP by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at 80% confluence in a six-well plate. Stable transformants were selected in medium containing 20 mg/L G418 for 4 wk. The abundance of HLCS/GFP fusion proteins was assessed in cytoplasm and nucleus from stably transfected cell lines. Briefly, cells were trypsinized and lysed, and proteins were resolved by gel electrophoresis using NuPage 4–12% Bis-Tris gels [27]. Transblots were probed using mouse anti-GFP (Roche Applied Science, Mannheim, Germany) and fluorophore-labeled donkey anti-mouse IgG in an Odyssey Infrared Imaging system (Licor, Lincoln, NE).

Subcellular fractionation

Previous studies suggest that the vast majority of HLCS localizes in cytoplasm and nucleus [5; 11; 24; 25; 28]. Briefly, ~10⁶ HEK-293 were scraped off the plastic surface and collected in 3 mL phosphate-buffered saline (4°C). Cytoplasmic and nuclear fractions were collected by using a commercial kit as described by the manufacturer (Nuclear Extract Kit; Active Motif, Carlsbad, CA). Contamination of nuclear extracts with cytoplasmic proteins was formally excluded by using chicken anti-GFP antibody as an exogenous tracer as follows. About 10⁶ HEK-293 cells were collected by scraping and resuspended in 3 ml of ice-cold phosphate-buffered saline/phosphatase provided with the Nuclear Extract kit. Cells were collected by centrifugation at 4°C for 5 min. The pellet was resuspended in 500 µl 1X Hypotonic Buffer from the kit. Chicken anti-GFP antibody (2.5µg) (Aves Labs, Tigard, Oregon) was added to the suspension and placed on ice for 15 min followed by adding 25 µl detergent provided in the kit and vortexing for 10 s. The mixture was centrifuged at 14,000 g for 30 s at 4°C. The cytoplasmic fraction in the supernatant was frozen at –80°C. The nuclear pellet was resuspended in 50 µl of Complete Lysis Buffer in the kit and vortexed for 10 s, incubated for 30 min on ice on a rocking platform. The suspension was vortexed for 30 s and centrifuged for 10 min at 14,000 g in a microcentrifuge at 4°C. The supernatant (nuclear fraction) was transferred into a pre-chilled microcentrifuge tube and stored at –80°C. Possible contamination in nuclear extraction from the fraction of cytoplasm was traced using IRDye 800CW Donkey Anti-Chicken IgG (Licor).

Confocal microscopy

HEK-293 cells transformed with HLCS-GFP plasmids were grown on microscope cover slips in six-well plates. The nuclear compartment was stained with 4',6-diamidino-2-

phenylindole (DAPI) Samples were analyzed using an Olympus FV500 confocal microscope (Microscopy Core Facility, University of Nebraska-Lincoln).

Carboxylase streptavidin blots

Carboxylase-bound biotin was probed as previously described [29]. Propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase contain biotinylated α subunits and nonbiotinylated β subunits; only α subunits are detectable in streptavidin blots.

Recombinant HLCS (rHLCS)

Full-length rHLCS was expressed and purified by using plasmid HCS-pET41a as previously described [13]. This plasmid codes for HLCS tagged with N-terminal glutathione S-transferase (GST), S-tag, and a 6 $\times$ his-tag, and a C-terminal 6 $\times$ his-tag (~115 kDa). N-terminally truncated rHLCS spans amino acids 58–722 and was prepared by PCR amplification of full-length HLCS with primers 5'-GTCCGAATTTCGGGGAGCATGTTGGCAGAG-3' (forward) and 5'-ATTCTCGAGCCCGCCGTTTGGGGAG-3' (reverse). The PCR product was digested with *EcoRI* and *XhoI* and cloned into vector pET41a (Novagen, Madison, WI). The plasmid was named "HLCS58-pET41a" and codes for a tagged HLCS (~108 kDa) with the same tags as in full-length rHLCS; its identity was verified by sequencing. Truncated HLCS58 was expressed and purified as described for full-length rHLCS [13]. Purities and identities of rHLCS and rHLCS58 were confirmed by gel electrophoresis, Coomassie blue staining, anti-His-tag antibody (Novagen), and an antibody to the C-terminus in human HCS [11].

Biotinylation of proteins by rHLCS58 *in vitro*

Here we tested whether HLCS58 has biotin ligase activity with regard to carboxylases and histones. The polypeptide p67 comprises the 67 C-terminal amino acids in human propionyl-CoA carboxylase including the biotinylation site lysine-669, and is a widely used substrate for assessing HLCS activity [2; 30]. We used biotin-free, recombinant p67 as substrate in HLCS58 activity assays as previously described for full-length HLCS [12]. Briefly, 0.3 μ g of p67 was incubated with 0.40 μ g of rHCS in 50 μ l of 75 mM Tris acetate buffer (pH 7.5), containing 0.3 mM biotin, 0.3 mM DTT, 7.5 mM ATP, and 45 mM MgCl₂ at 37°C for 2 h. p67-bound biotin was visualized by gel electrophoresis [26], using IRDye 800CW Labeled Streptavidin and an Odyssey Infrared Imaging system (Licor) [16].

Biotinylation of histones by rHCS58 was tested as described for p67 with the following modifications. Ten micrograms each of recombinant human histones H2A, H2B, H3.2 or H4 (New England Biolabs, Ipswich, MA), were substituted for p67; the amount of rHLCS58 in reaction mixtures was increased to 1.0 μ g, and incubations were conducted for 12 h. Reactions were terminated by adding Tris-Glycine loading buffer (Invitrogen) and heating at 95°C for 10 min. Proteins were resolved using 18% Tris-Glycine gels, and protein-bound biotin in transblots was probed with IRDye 800CW Labeled anti-biotin (Abcam, Cambridge, MA).

RESULTS

Translation of HLCS58 in HEK-293 cells

Studies with HLCS-GFP fusion vectors confirmed that methionine-58 can serve as a translational start site. When HEK-293 cells were transformed with HLCS-GFP (Fig. 1A), both full-length HLCS and HLCS58 were detectable by using anti-GFP as probe; methionine-1 was preferred as translational start site compared with methionine-58 (Fig. 1B, left lane). Note that these analyses were conducted in cytoplasmic extracts, and that shuttling of HLCS58 into the nucleus could also explain the relatively low abundance of that variant

in the cytoplasm (see below). When cells were transformed with vector HLCS58-GFP (Fig. 1A), methionine-58 became the only available translational start site, which was readily utilized for expression of HLCS (Fig. 1B, positive control). In contrast, when cells were transformed with the methionine-58 mutant vector HLCS58m-GFP (Fig. 1A), methionine-1 became the only available translational start site, which was readily utilized for expression of HLCS (Fig. 1B, negative control). The nucleotide sequences surrounding the first ATG (methionine-1, CATGG) and the third ATG (methionine-58, ATGG) both fit the Kozak consensus sequence for translation start sites [31] (Fig. 1A). Methionine-58 is conserved in HLCS from humans, chimpanzees, and mice, but is not present in HLCS from chicken and zebrafish (Fig. 1C).

HLCS58 is a nuclear protein

HLCS58 localizes in both cytoplasmic and nuclear compartments, whereas only traces of full-length HLCS can be found in the nucleus. First, HEK-293 cells were transformed with vectors HLCS58m-GFP and HLCS58 to express GFP fusion protein of full-length HLCS and HLCS58, respectively. Analysis by confocal microscopy suggests that only the N-terminally truncated variant can be found in the nuclear compartment (Fig. 2A,B). Second, HE K-293 cells were transformed with all three HLCS vectors and nuclear and extracts were probed with anti-GFP (Fig. 2C). Traces of full-length HLCS were detected in the nuclear compartment (left lane), whereas substantial amounts of HLCS58 were detected in the nucleus (middle lane). If translation from methionine-58 blocked was prevented by transformation with the Met58Leu mutant HLCS, the protein localized in the nucleus as well (right lane). Equal loading was confirmed using an antibody to the nuclear protein histone H3 (bottom gel). Previous studies by us and others [25] suggest that the abundance of endogenous HLCS is low and, therefore, that endogenous full-length HLCS cannot be detected in the cell nucleus. However, upon overexpression, small amounts of full-length HLCS become detectable in the nucleus (Fig. 2C, left lane). We formally excluded the possibility of nuclear extracts with cytoplasmic proteins by adding chicken anti-GFP as a tracer to whole HEK-293 lysates prior to isolation of nuclear material. The immunoglobulin was detectable exclusively in the cytoplasmic fraction (Fig. 2D).

Carboxylase biotinylation increased for 3-methylcrotonyl-CoA carboxylase in HLCS overexpression cells

Overexpression of both full-length HLCS and HLCS58 caused only a moderate increase in the overall biotinylation of carboxylases compared with wild-type cells. HEK-293 cells were transformed with vectors HLCS58m-GFP and HLCS58 to express GFP fusion protein of full-length HLCS and HLCS58, respectively. Analysis by streptavidin blots suggests that carboxylase biotinylation increased to a meaningful extent only for the α subunit of 3-methylcrotonyl-CoA carboxylase compared with wild-type HEK-293 cells (Fig. 3).

Biotin protein ligase activity of HLCS58

HLCS58 has catalytic activity to mediate biotinylation of both carboxylases and histones *in vitro*. In previous studies we devised a protocol for preparing catalytically active full-length rHLCS, which takes advantage of an expression system that facilitates protein folding [13]; this protocol yields rHLCS of much greater specific activity than those prepared by other published protocols (unpublished observation). First, the identities and purities of tagged full-length rHLCS (~115 kDa) and rHLCS58 (~108 kDa) were confirmed by staining with coomassie blue and probing with ant-his tag and anti-HLCS (Fig. 4A). Unexpectedly, transformation of *E. coli* with plasmid HCS-pET41a produced a faint band of 108 kDa in addition to a strong band of 115 kDa in samples probed with coomassie blue and anti-his. The 108-kDa band co-migrated with the band produced by rHLCS58 from cells transformed

with HLCS58-pET41a, suggesting that *E. coli* might be able to use the methionine-58 start codon.

rHLCS58 was catalytically active, judged by its ability to biotinylate both p67 and four classes of histones (Fig. 4B,C); negative controls were created by omitting rHLCS58 or substrate, and equal loading was confirmed by using coomassie blue.

DISCUSSION

It has long been suspected that translation of HLCS may start at methionines-1, -7, and -58 [23; 25]. This paper offers the following novel insights into HLCS biology. First, by mutating methionine-58 to leucine we provided unambiguous evidence that methionine-58 serves as an in-frame alternative translation site for HLCS transcripts. Second, we added evidence to a recent report suggesting that HLCS58 is a nuclear protein [25]. Third, full-length HLCS may also enter the nucleus, at least in transformed cell lines overexpressing the protein. Fourth, we provide unambiguous evidence that HLCS58 is catalytically active. Fifth, in previous studies we demonstrated that some of the N-terminal 446 amino acids are important for p67/HLCS interactions [32], which was confirmed by others [33]. Those observations, combined with this report, suggest that N-terminal domains between methionine-58 and phenylalanine-446 are important for interactions between HLCS and its substrates, and that the N-terminal 57 amino acids might not be essential for substrate recognition.

Nuclear localization of HLCS has been reported before [5; 6; 11], but a recent report [25] suggests that the abundance of nuclear HLCS might have been overestimated due to lack of specificity of an antibody used in one of these studies [5]. The report by Bailey *et al.* further suggests that antibodies in other studies might also lack specificity [25]. We endorse the concerns voiced by Bailey *et al.* with regard to antibody specificities, which could be particularly problematic because of the low abundance of endogenous HLCS [25]. Even though Bailey did not re-examine our antibody to HLCS58, we are currently in the process of developing a monoclonal antibody, which might help to address some of these concerns.

With the recent report by Bailey *et al.* and this report, there is now sufficient evidence to suggest that biologically active HLCS58 enters the nuclear compartment [25]. Our data suggest that full-length HLCS may also enter the nuclear compartment. Evidence further suggests that chromatin-bound HLCS might accumulate in the nuclear lamina [5]. This raises the important question as to what the biological function of nuclear HLCS might be. Clearly, rHLCS has catalytic activity to biotinylate histones *in vitro* [13]. On the other side, we suggested early on that biotinylation of histones is a rare event *in vivo* based on radiotracer studies [8], which was subsequently re-emphasized by Bailey *et al.* [34] and possibly overstated by Healy *et al.* [35]. Our repeated observations that biotinylated histones are enriched in repressed loci and repeat regions [15; 16; 17; 36] are consistent with the following revised model to explain roles of HLCS in gene regulation and genome stability. We propose that HLCS is part of a larger multiprotein complex in chromatin that participates in gene repression. Interactions with other chromatin proteins would explain the punctuate pattern of HLCS binding to chromosomes [6]. HLCS/protein interactions would also explain the non-random, low-abundance biotinylation of histones in chromatin observed by us and others. We have already identified candidate proteins for interactions with HLCS (see Introduction) and are actively pursuing this line of research.

The identification of HLCS-interacting proteins might also help with explaining how HLCS enters the nucleus despite lacking a nuclear localization signal. One can easily envision scenarios where proteins with nuclear localization signals facilitate the nuclear import of

HLCS. Histone H3 has a strong nuclear localization signal and is known to interact with HLCS [13]. Therefore, histone H3 might be one possible candidate for facilitating the nuclear import of HLCS.

Highlights

- Unambiguous evidence is provided that methionine-58 serves as an in-frame alternative translation site for holocarboxylase synthetase (HLCS) transcripts.
- Both full-length HLCS and HLCS enter the nuclear compartment, but HLCS is the predominant nuclear variant.
- HLCS58 has biological activity as biotin protein ligase.

Abbreviations

GFP	green fluorescent protein
HLCS	holocarboxylase synthetase
HLCS58	HLCS with a translation start site in methionine-58
rHLCS	recombinant human holocarboxylase synthetase
DAPI	4',6-diamidino-2-phenylindole

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Fig. 1. Alternative translation sites for HLCS in HEK-293 cells

(A) Schematic presentation of vectors HLCS-GFP, HLCS58-GFP, and HLCS58m-GFP. The Kozak consensus sequences are underlined. (B) HLCS-GFP, HLCS58-GFP and HLCS58m-GFP in cytoplasmic extracts from HEK-293 cells were probed using anti-GFP. (C) Conservation of alternative translation sites of HLCS [accession numbers NM_000411.4 (*Homo sapiens*), XM_531454.2 (*Pan troglodytes*), NM_139145.3 (*Mus musculus*), XM_416725.2 (*Gallus gallus*) and XM_001919110.1 (*Danio rerio*)]. The nucleotides coding for Met-1, Met-7, and Met-58 are highlighted by underlining, dashed underlining, and double underlining, respectively.

Fig. 2. HLCS58 enters HEK-293 cell nuclei

Cells were transformed with HLCS58m-GFP (panel A) or HLCS58 (panel B), and GFP was visualized by confocal microscopy (green). Nuclei were stained with DAPI (blue). The right column shows the merged GFP and DAPI tracer images. Nuclear extracts of HEK-293 cells transformed with HLCS-GFP, HLCS58-GFP, or HLCS58m-GFP were probed with anti-GFP (panel C, upper panel) and anti-histone H3 (lower panel). Absence of cytoplasmic contaminants in nuclear extracts was monitored by using exogenous chicken anti-GFP (Ig) as tracer (panel D).

Fig. 3. Biotinylation of carboxylases in transgenic HEK-293 cells

Streptavidin was used to probe biotin in acetyl-CoA carboxylase (ACC), pyruvate carboxylase (PC), and the α -chains of 3-methylcrotonyl-CoA carboxylase (MCC) and propionyl-CoA carboxylase (PCC).

Fig. 4. Catalytic activities of full-length rHLCS and rHLCS58

(A) Full-length rHLCS (~115 kDa) and rHLCS58 (~108 kDa) were probed with coomassie blue, anti-his tag, and anti-HLCS. (B) rHLCS58 was incubated with p67; p67-bound biotin was probed using streptavidin. (C) rHCS58 was incubated with recombinant human histones H2A, H2B, H3.2 or H4; histone-bound biotin was probed using anti-biotin. Equal loading of histones was confirmed by staining with coomassie blue.