short communication

Cloning and characterization of a cDNA encoding human cardiolipin synthase (hCLS1)

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Abstract Cardiolipin (CL) is a phospholipid localized to the mitochondria, and its biosynthesis is essential for mitochondrial structure and function. We report here the identification and characterization of a cDNA encoding the first mammalian cardiolipin synthase (CLS1) in humans and mice. This cDNA exhibits sequence homology with members of a CLS gene family that share similar domain structure and chemical properties. Expression of the human CLS (hCLS1) cDNA in reticulocyte lysates or insect cells led to a marked increase in CLS activity. The enzyme is specific for CL synthesis, because no significant increase in phosphatidylglycerol phosphate synthase activity was observed. In addition, CL pool size was increased in hCLS1-overexpressing cells compared with controls. Furthermore, the hCLS1 gene was highly expressed in tissues such as heart, skeletal muscle, and liver, which have been shown to have high CLS activities. These results demonstrate that hCLS1 encodes an enzyme that synthesizes CL.—Lu, B., F. Y. Xu, Y. J. Jiang, P. C. Choy, G. M. Hatch, C. Grunfeld, and K. R. Feingold. **Cloning and characterization of a cDNA encoding human cardiolipin synthase (hCLS1). *J. Lipid Res.* 2006.** 47: **1140–1145.**

Supplementary key words mitochondria • phospholipid • diphosphatidylglycerol

Cardiolipin (CL) is a phospholipid localized to the mitochondria (1), where it plays a key role in mitochondrial function (2, 3). In eukaryotic cells, CL is predominantly present in mitochondrial inner membranes (4, 5), where it provides osmotic stability to membranes and interacts with several key mitochondrial enzymes for their optimal oxidative activities (6, 7). In mammals, CL is thought to be required for many functions. For example, alterations in the content and the acyl composition of CL result in a mitochondrial structural abnormality (8, 9) and reduced oxygen consumption (10). Moreover, a reduction in CL is a key abnormality both in Barth syndrome (11), an X-linked genetic disease, and in animal models (12, 13). Although the exact role that CL plays in the apoptotic process remains controversial (14, 15), data suggest that alterations in CL metabolism are involved in regulating mammalian aging (12) and cell death (16). Thus, maintenance of CL is required for mammalian cell function.

In mammalian tissue, CL and its synthetic enzyme cardiolipin synthase (CLS) are located exclusively in mitochondria. De novo CL biosynthesis occurs on the matrix side of the inner mitochondrial membrane (17) via the cytidine-5′diphosphate-1,2-diacylglycerol (CDP-DG) pathway [reviewed in (4)]. Phosphatidic acid is converted to CDP-DG by CDP-DG synthase. The CDP-DG formed then condenses with glycerol-3-phosphate to form phosphatidylglycerol (PG) catalyzed by phosphatidylglycerol phosphate (PGP) synthase and PGP phosphatase. In the final step, PG is converted to CL by condensation with CDP-DG catalyzed by the terminal enzyme CLS.

Although a mammalian CLS has been purified to homogeneity (18), its gene has not been identified. Through homology searches of the expressed sequence tag (EST) databases with the yeast CLS, we identified a UniGene on human chromosome 20, (C20orf155). In this study, we demonstrate that C20orf155 encodes a human CLS, now termed hCLS1. The expression of hCLS1 cDNA in vitro and in vivo resulted in high levels of CLS activity, supporting its role as a CLS.

MATERIALS AND METHODS

Materials

[α-^{32}P]dCTP was purchased from Perkin-Elmer Life Sciences (Boston, MA). TLC (silica gel 60) plates were from Fisher Scien-

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Abbreviations: CDP-DG, cytidine-5′diphosphate-1,2-diacylglycerol; CL, cardiolipin; CLS, cardiolipin synthase; EST, expressed sequence tag; PG, phosphatidylglycerol; PGP, phosphatidylglycerol phosphate.

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tific. Lipid standards were from Serdary Research Laboratories. All other biochemicals were from Sigma or Fisher Scientific.

Insect cell culture and transfection

Insect Sf9 cells were from Invitrogen. Cells were grown in 10 cm dishes in Grace's Insect Medium supplemented with 10% fetal calf serum at 27°C. For transfection, recombinant viral DNA in Cellfectin Reagent was added to Sf9 cells according to the Baculovirus Expression Systems protocol (Invitrogen). After transfection, P1 viral stock was collected.

Northern blot analysis

Northern blots containing poly(A) RNA from selected human tissues were purchased from Clontech (Palo Alto, CA). Hybridization was performed using [α-^{32}P]dCTP-labeled hCLS1 probes as described (19).

PCR and double-stranded DNA sequencing

The coding region of hCLS1 was amplified with a pair of specific primers (**Fig. 1A**). PCR was performed using Platinum Pfx DNA polymerase (Invitrogen). Sequencing analysis was performed at the Institute of Cell Biology in Winnipeg, Canada.

Identification and cloning of mammalian CLS cDNAs

Yeast CLS protein sequences were used to search against the translated est_human database. More than 100 BLAST hits revealed one unique gene, C20orf155. One EST clone (clone identifier 2369931; Invitrogen) containing hCLS1 cDNA was purchased and sequenced. The full-length coding sequences were amplified using Platinum Pfx DNA polymerase (Invitrogen). The amplified DNA fragment was subcloned into an expression vector, pcDNA3.1/V5-His (Invitrogen). Similarly, mouse CLS (mCLS1) cDNA was obtained and engineered into pcDNA3.1/V5-His vector.

In vitro transcription and translation

The in vitro transcription and translation reactions were performed using a coupled transcription and translation system (TnT Coupled Reticulocyte Lysate System; Promega), as described previously (20).

Insect cell expression studies

The hCLS1/mCLS1 sequences were first subcloned into pENTR and subsequently into baculovirus vector according to the manufacturer's manual (Invitrogen). Recombinant viruses were generated by transfecting Sf9 cells and selecting with ganciclovir. High viral titers were obtained from three rounds of amplification. P3 recombinant viruses were used to express recombinant hCLS1/mCLS1 in Sf9 cells.

Western blot analysis for recombinant hCLS1/mCLS1

Samples containing ~10–20 μl of reticulocyte lysates or ~80–100 μg of cell extracts from virus-infected Sf9 cells were subjected to SDS-PAGE as described (20). The protein fractions in the gel were transferred onto polyvinylidene difluoride membranes, which were incubated with conjugated anti-V5 antibodies (1:5,000 dilution; Invitrogen). The protein bands were detected using the SuperSignal Extended Duration Substrate Kit (Pierce, Rockford, IL).

Enzyme assays and pool sizes of phospholipids

PGP synthase and CLS enzyme assays were measured using methods described previously (21). The pool sizes of major phospholipids were determined in control and hCLS1-overexpressing Sf9 cells as described (21).

Statistical analysis

Results are presented as means ± SEM. Statistical significance between two groups was determined by Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

hCLS1 enzyme protein, gene locus, and tissue expression pattern

Through homology searches of the EST databases using yeast CLS, we identified a cDNA encoding putative hCLS1. The translation of hCLS1 predicts a 301 amino acid protein that exhibits 34% sequence identity to yeast CLS sequences (162 amino acids near the C terminus) (Fig. 1A).

Analysis of hCLS1 protein sequences revealed two critical features. At its N terminus, hCLS1 exhibited a preponderance of basic and hydroxyl-carrying residues, suggesting mitochondrial target signaling (22) (Fig. 1A). This mitochondrial target signaling of hCLS1 was also predicted by a subcellular localization prediction program (TargetP1.1 server at http://www.cbs.dtu.dk/cgi-bin/). At its C terminus, a significant sequence alignment with both CDP-alcohol phosphatidyltransferase (pfam01066; CD-Length = 145 residues, 83.4% aligned) and PGP synthase (COG0558; CD-Length = 192 residues, 80.7% aligned) was revealed, suggesting an enzyme containing CDP-alcohol phosphatidyltransferase activity (Fig. 1A).

The hCLS1 EST sequences were mapped to a region of human chromosome 20, C20orf155 (GenBank accession number 27501067). Because the EST sequences are mapped to the chromosome DNA, hCLS1 is transcribed from human genome, but not mitochondrial genome. As shown in Fig. 1B, the hCLS1 probe hybridized primarily with two dominant mRNA transcripts at ~1.6 and ~2.2 kb as well as with a minor transcript at ~4.1 kb. hCLS1 was highly expressed in heart, skeletal muscle, and liver; low to moderate levels were seen in pancreas, kidney, and placenta; and very low levels were found in lung and brain. This tissue expression pattern correlates well with CLS activity in these tissues (4).

hCLS1 belongs to the CLS gene family

Through homology searches of nonredundant databases at the National Center for Biotechnology Information, the mouse (AAH48702) and rat (NP_0014280) CLS sequences were also identified. rCLS1 and mCLS1 shared 85% and 86% amino acid sequence identity with hCLS1, fulfilling the criteria for counterparts in different species. To define the sequence conservation among CLS family members, eukaryotic CLS proteins were analyzed with the ClustalW alignment program (**Fig. 2A**). Although the overall matches between members varied (21–97%), the region near the protein C terminus is largely conserved. Within this region, four highly conserved stretches/motifs were apparent: a CDP-OH-P motif, $D(X)_2DG(X)_2AR(X)_{8\text{-}9}$

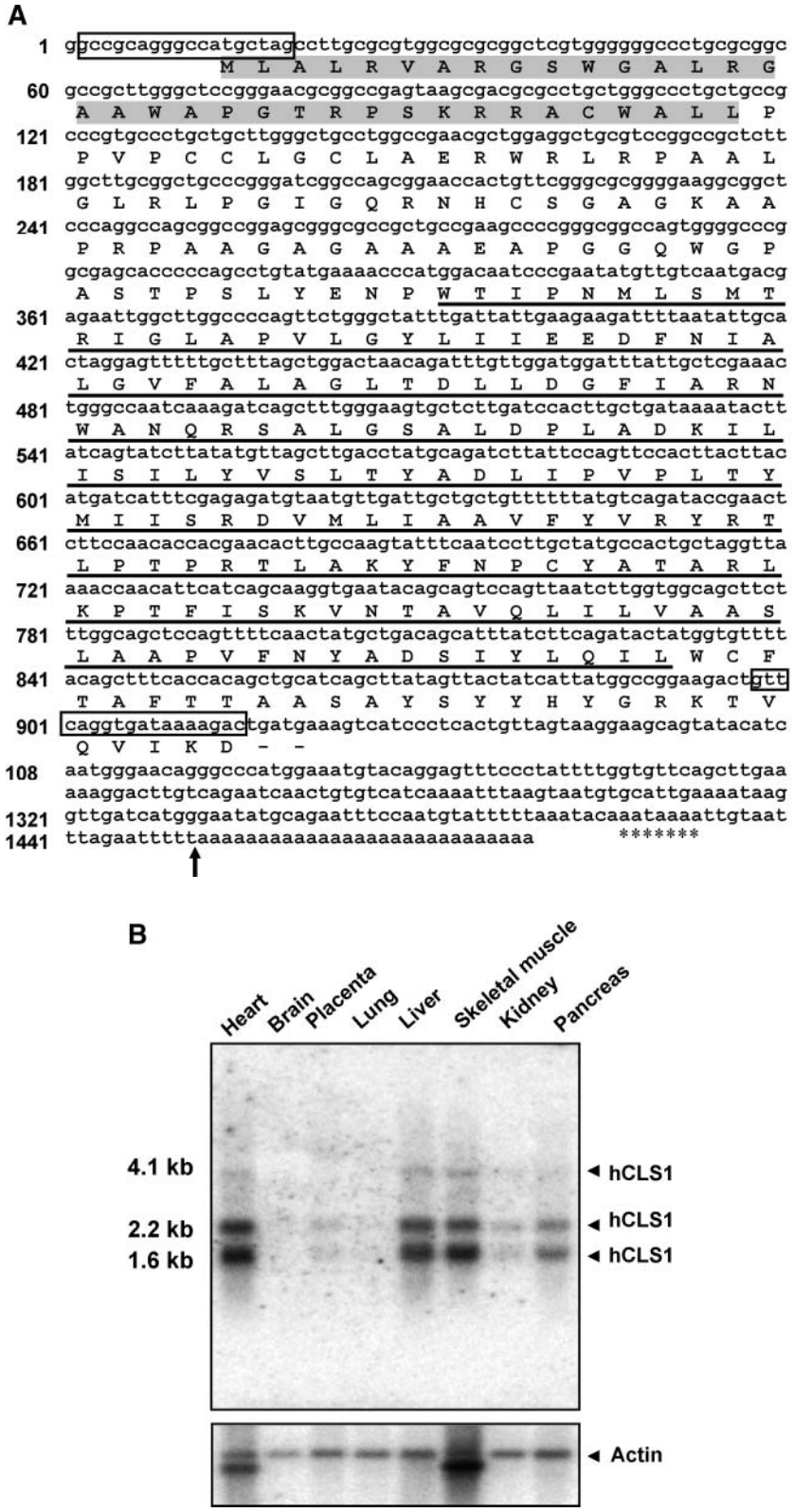

Fig. 1. The human cardiolipin synthase protein (hCLS1) and its tissue expression pattern. A: Features of the predicted peptide sequence of hCLS1. A mitochondrial targeting sequence at the N terminus is shaded. The region conserved among proteins containing a CDP-alcohol phosphatidyltransferase domain (KOG1617; pfam01066) or a PGP synthase domain (COG0558) is underlined. The poly(A) signal is indicated with asterisks. The arrow indicates the cleavage and a poly(A) tail. The boxed nucleotide sequences are the primers used to amplify the coding cDNA. B: Tissue expression pattern of hCLS1. Human multiple tissue blots containing 2 μg of poly(A)$^+$ RNA for each tissue were hybridized with radiolabeled probes prepared from the full-length cDNA of hCLS1. The hybridized blots were stripped of residual radioactivity and reprobed with β-actin cDNA as an internal control.

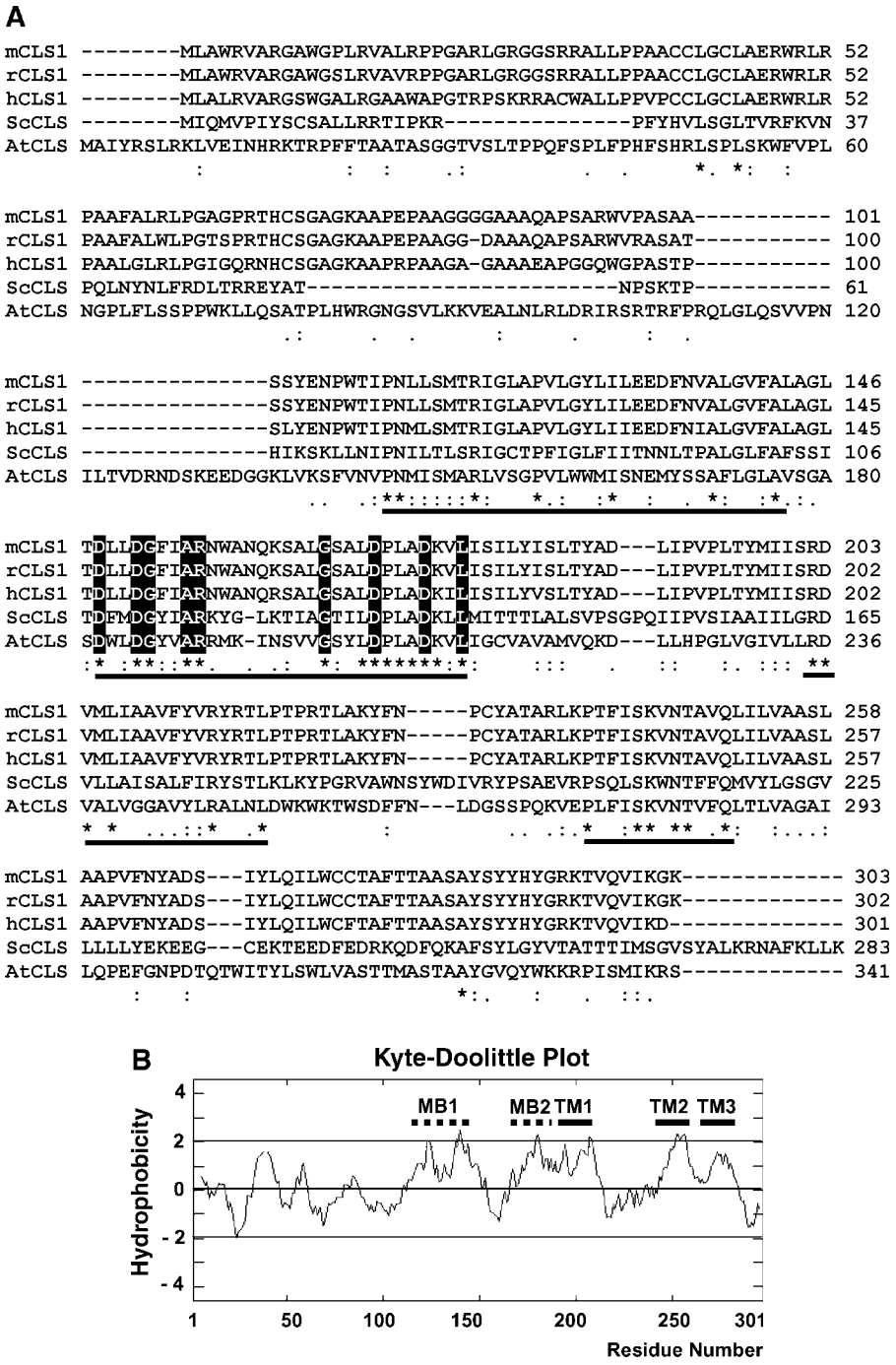

Fig. 2. Sequence comparison of members of the CLS gene family. A: Alignment of predicted mammalian CLS protein sequences (human, rat, mouse) with those of plant and yeast CLS. The protein sequences were from hCLS1 (NP_061968), mouse CLS1 (mCLS1; AAH48702), rat CLS1 (rCLS1; NP_0014280), plant *Arabidopsis thaliana* CLS (AtCLS; NP_567273), and yeast *Saccharomyces cerevisiae* CLS (ScCLS; NP_010139.1). Dashes represent gaps to maximize alignment of the sequences. Amino acid residues identical for all eukaryotic CLS proteins are indicated with asterisks. Conservation of conserved substitutions is indicated with two dots, and semiconserved substitutions are indicated with one dot. The amino acid residues conserved with the CDP-OH-P motif are shaded in black. Four novel conserved motifs are underlined. B: Hydrophobicity plot of hCLS1 as assessed by Kyte-Doolittle analysis. The thick solid lines indicate three predicted transmembrane helices (TM1–TM3), and the thick dashed lines indicate two membrane binding regions (MB1 and MB2).

$G(X)_3D(X)_3D(X)_2L$, which is necessary for CLS enzyme activity (23), and three other novel motifs that may be important for CLS structure and function (23). Because all eukaryotic CLS proteins identified to date have multiple transmembrane domains at the C terminus, we examined whether hCLS1 also has this feature. Analysis by transmembrane domain programs (http://www.cbs.dtu.dk/services/TMHMM/) found two hydrophobic domains (amino acid

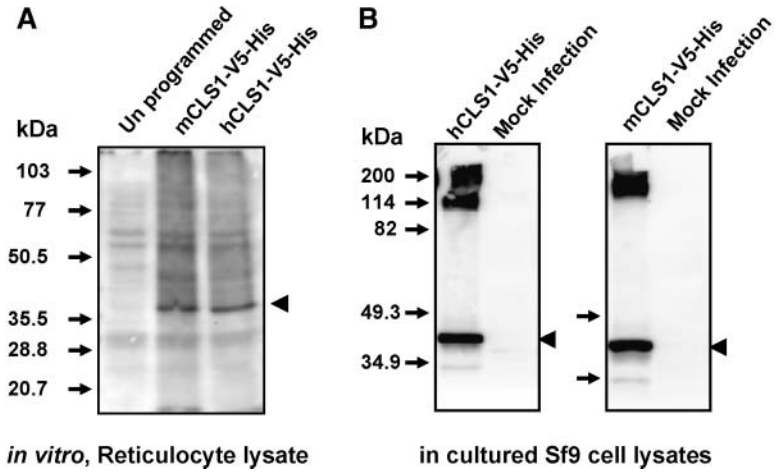

Fig. 3. Expression of human and mouse CLS1 in vitro and in vivo. Expression of V5-tagged hCLS1/mCLS1 in vitro in reticulocyte lysate (A) and in cultured Sf9 cells (B). The protein products were detected by Western blot analysis, as described in Materials and Methods. The high molecular mass band may represent the aggregation of CLS protein or its association with other proteins under our experimental conditions.

positions 120–138 and 178–189) and three transmembrane helices (amino acid positions 190–212, 240–262, and 267–289) (Fig. 2B). This topology is consistent with previous findings that CLS has multiple membrane domains overlapping its catalytic region (23).

Mammalian CLS1 encodes a 32 kDa protein

To examine whether our cloned mammalian CLS1 has the ability to encode a protein, mCLS1 and hCLS1 were expressed in two experimental systems. First, expression of both V5-His-tagged mCLS1 and hCLS1 was performed in rabbit reticulocyte lysates using an in vitro coupled transcription and translation system. Expression of tagged hCLS1/mCLS1 was analyzed by Western blot using a V5-epitope antibody. A major protein band of ~38 kDa was found for tagged hCLS1/mCLS1 (**Fig. 3A**); therefore, the molecular mass of wild-type hCLS1/mCLS1 (without the V5-His tag) would be ~32 kDa, which is consistent with their calculated mass. To confirm this result, expression of the tagged hCLS1/mCLS1 was conducted in Sf9 cells. Similar to the in vitro translation finding, a protein band at ~38 kDa was detected in the transfected cell lysates by Western blot analysis (Fig. 3B). Thus, both hCLS1 and mCLS1 could be expressed under our experimental conditions.

hCLS1 exhibits specific CLS activities

CLS enzyme activity in vitro and in cultured cells was determined. In the programmed reticulocyte lysates, hCLS1 expression increased CLS activity by >40-fold over control (**Fig. 4A**). Furthermore, when recombinant hCLS1 was expressed in Sf9 insect cells, mitochondrial membrane CLS activity was found to be >5-fold higher than that in membranes from controls (Fig. 4B). In addition, analysis of phospholipid mass from these cells showed an increase in CL of ~25% (3.54 ± 0.75 vs. 2.81 ± 0.6 nmol/mg proteins; two measurements), whereas no increase was seen with other phospholipids (data not shown). The fact that little endogenous CLS activity was found in unprogrammed (without expression plasmid DNA input) reticulocyte lysates (lacking mitochondria) strongly supports the notion that the hCLS1 gene encodes a CLS enzyme, not a cofactor of a CLS enzyme.

Given the 80.7% alignment with the conserved domain of PGP synthase, PGP synthase activity was also measured in the membrane fractions of Sf9 cells overexpressing hCLS1. PGP synthase activity was not increased significantly in cells overexpressing hCLS1 (9.09 ± 0.49 vs. 8.98 ± 1.16 pmol/mg/min in controls), indicating the specificity of hCLS1 for the biosynthesis of CL.

DISCUSSION

It is well documented that CL biosynthesis occurs on the matrix side of the inner mitochondrial membrane via the CDP-DG pathway (17). In the final step, CLS catalyzes the transfer of a phosphatidyl moiety from CDP-DG to PG to form CL. Although a rat CLS enzyme has been purified to homogeneity (18), a gene encoding the enzyme has not been identified.

In this study, we identified a cDNA that encodes the first mammalian CLS enzyme, termed CLS1, in humans and mice. Evidence that CLS1 is a CLS enzyme is provided by the following findings. First, expression of hCLS1 cDNA in reticulocyte lysates resulted in an ~40-fold increase in

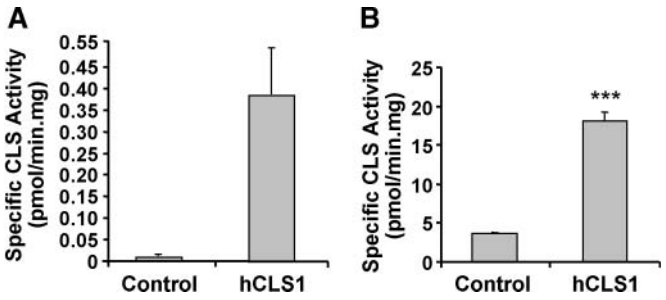

Fig. 4. Characterization of hCLS1 enzyme activity. CLS enzyme activity assay was performed as described in Materials and Methods. A: CLS enzyme activity in unprogrammed reticulocyte lysates (control) or programmed lysates expressing hCLS1. Results are means ± SEM of two measurements. B: CLS enzyme activity in mitochondrial membrane fractions from either control cells or Sf9 cells overexpressing hCLS1. Results are means ± SEM of three measurements. *** $P < 0.001$.

radiolabeled CL product (Fig. 4A). Second, expression of hCLS1 cDNA in Sf9 cells resulted in a significant increase (~5-fold) in CLS activities. In addition, this increase in CLS activities resulted in an increase in CL pool size in hCLS1-overexpressing cells. Third, the expressed hCLS1 displayed specificity for CL synthesis, because no significant increase in PGP synthase activities was observed. Finally, high levels of hCLS1 transcripts were found in tissues (Fig. 1B) that have both high mitochondrial content and CLS activities (4).

Mammalian CLS1 exhibits a convergence in its domain structure and molecular properties. All eukaryotic CLS proteins are integral membrane proteins with an invariable domain topology: an N-terminal mitochondrial target sequence followed by two membrane binding/associating domains and three transmembrane helices. Interestingly, the CDP-OH-P motif that is required for CLS activity also invariably resides in the membrane binding/associating regions (Fig. 2). This topology supports a model in which CL is synthesized in the matrix leaflet of the inner membrane near the membrane/water interface (17). Other conserved features include a high isoelectric point (pH 9.7–9.9) and a molecular mass of ~32 kDa. In addition to these features, the hCLS1 was most abundantly expressed in insulin-responsive and mitochondria-enriched tissues such as heart, skeletal muscle, and liver. These data strongly suggest that the cloned mammalian CLS1 is an evolutionarily conserved enzyme that is highly expressed in mitochondria-enriched tissues.

In summary, we have identified and characterized a gene encoding the first mammalian CLS in human, rat, and mouse. Because CLS1 catalyzes the terminal step for CL formation, the requirement for CL in mitochondrial function at the molecular level can now be more precisely defined. Likewise, regulation and expression of CLS1 can be used to define the role of CL in apoptosis. Finally, the discovery of the hCLS1 gene has laid a foundation for future studies to determine whether CLS is a feasible target for promoting CL biosynthesis, thereby treating human diseases such as Barth syndrome.

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