Published in final edited form as:

Biochim Biophys Acta. 2010 April ; 1801(4): 438–445. doi:10.1016/j.bbalip.2009.12.002.

A Novel Function of the Human CLS1 in Phosphatidylglycerol Synthesis and Remodeling

Jia Nie^{1,3,‡}, Xinbao Hao^{1,‡}, Daohong Chen², Xiao Han³, Zhijie Chang⁴, and Yuguang Shi^{1,#}

¹ Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, 500 University Drive, H166, Hershey, PA 17033

² Cancer Research, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285

³ Key Laboratory of Human Functional Genomics of Jiangsu Province, Nanjing Medical University, Nanjing, China

⁴ School of Medicine, Institute of Biomedicine, State Key Laboratory of Biomembrane and Membrane Biotechnology, Tsinghua University, Beijing, China

Abstract

Phosphatidylglycerol (PG) is a precursor for the biosynthesis of cardiolipin and a signaling molecule required for various cellular functions. PG is subjected to remodeling subsequent to its *de novo* biosynthesis in mitochondria to incorporate appropriate acyl content for its biological functions and to prevent the harmful effect of lysophosphatidylglycerol (LPG) accumulation. Yet, a gene encoding a mitochondrial LPG acyltransferase has not been identified. In this report, we identified a novel function of the human cardiolipin synthase (hCLS1) in regulating PG remodeling. In addition to the reported cardiolipin synthase activity, the recombinant hCLS1 protein expressed in COS-7 cells and Sf-9 insect cells exhibited a strong acyl-CoA dependent LPG acyltransferase activity, which was further confirmed by purified hCLS1 protein overexpressed in Sf-9 cells. The recombinant hCLS1 displayed an acyl selectivity profile in the order of C18:1 > C18:2 > C18:0 > C16:0, which is similar to that of hCLS1 toward PGs in cardiolipin synthesis, suggesting that the PG remodeling by hCLS1 is an intrinsic property of the enzyme. In contrast, no significant acyltransferase activity was detected from the recombinant hCLS1 enzyme toward lysocardiolipin which shares a similar structure with LPG. In support of a key function of hCLS1 in PG remodeling, overexpression of hCLS1 in COS-7 cells significantly increased PG biosynthesis concurrent with elevated levels of cardiolipin without any significant effects on the biosynthesis of other phospholipids. These results demonstrate for the first time that hCLS1 catalyzes two consecutive steps in cardiolipin biosynthesis by acylating LPG to PG and then converting PG to cardiolipin.

INTRODUCTION

Cardiolipin (CL) is a major membrane polyglycerolphospholipid of the mitochondria, where it serves specific roles in mitochondrial structure and function. CL is required for the

#To whom correspondences should be addressed: Yuguang Shi, Ph.D., Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, 500 University Drive, H166, Hershey, PA 17033, Tel: 717-531-0003/Fax: 717-531-7667, yus11@psu.edu.

‡These authors contributed equally

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

reconstituted activity of a number of metabolic enzymes and carrier proteins in the mitochondria [1,2]. CL in the inner mitochondrial membrane serves as a Ca^{2+} -binding site, through which Ca^{2+} triggers mitochondrial membrane permeabilization [3]. Additionally, CL is a mediator for the onset of apoptosis by providing specificity for targeting of tBid to mitochondria and by interacting with cytochrome *c* [4–6]. In yeast *S. cerevisiae*, mutation of the *crd1* gene encoding a CL synthase results in impaired viability, a reduced membrane potential, and defective oxidative phosphorylation [7].

In eukaryotic mitochondria, CL is synthesized by three consecutive steps that begin with the synthesis of phosphatidylglycerol (PG) from cytidine diphosphate-diacylglycerol (CDP-DAG). CDP-DAG is generated from phosphatidic acid catalyzed by phosphatidic acid:CTP cytidyltransferase, and then converted to PG through the sequential enzymatic actions of phosphatidylglycerol phosphate (PGP) synthase and PGP phosphatase [8,9]. The committed and rate-limiting step in *S. cerevisiae* is catalyzed by PGP synthase (PGS) [10]. The final step of CL synthesis involves transfer of a phosphatidyl residue from CDP-diacylglycerol to PG, which is catalyzed by CL synthase (CLS).

PG is an important precursor for the synthesis of CL. Consequently, disruption of the *PGS1* gene in yeast causes PG and CL deficiency and inhibition of growth on nonfermentable carbon sources [7]. In yeast cells, PG appears to substitute for CL functions, as evidenced by a significant increase in PG content resulted from disruption of the *CRD1* gene that encodes CL synthase when grown in a nonfermentable carbon source [7,11]. PG deficiency in Chinese hamster ovary cells caused by a mutation in the *PGS1* gene results in CL deficiency and mitochondrial dysfunction manifested by increased glycolysis, reduced oxygen consumption, stringent temperature sensitivity for cell growth in glucose-deficient medium, and reduced ATP production [10].

As a precursor for CL synthesis, PG is subjected to remodeling after its *de novo* synthesis. We have recently cloned and characterized a gene encoding the first acyl-CoA dependent LPG acyltransferase (LPGAT1) that catalyzes acylation of LPG to PG [12]. Intriguingly, the LPGAT1 enzyme is localized in ER. PG remodeling is also believed to be carried out in the mitochondria where PG is synthesized. For example, an acyl-CoA dependent LPG acyltransferase activity has previously been reported in mitochondria [13], and displayed different acyl selectivity toward LPG from that of the LPGAT1 enzyme. However, little is known about the LPGAT isoform or the encoding gene involved in the PG remodeling process in the mitochondria.

Cumulative evidence suggests that PG remodeling and synthesis is coupled with the biosynthesis of CL. In rat hearts perfused with [^{14}C]-glycerol, there was a time-dependent accumulation of radioactivity incorporated into CL and LPG, but not other phospholipids involved in the biosynthesis of CL [14]. LPG has been shown to inhibit CLS activity from various organisms [15]. PG remodeling plays a role in the onset of Barth syndrome, an X-linked recessive disease caused by mutations of the *tafazzin* gene encoding a transacylase/acyltransferase involved in CL remodeling [16,17]. In cultured skin fibroblasts from patients of Barth syndrome, both PG and CL remodeling is defective, as evidenced by an decrease in CL synthesis and the incorporation of linoleic acid into both PG and CL. [18]. An increase in saturated fatty acid content in PG causes a decline in cardiolipin synthesis in cultured cardiomyocyte, and corresponds with cytochrome *c* release in palmitate-induced cardiomyocyte apoptosis [19]. In *S. cerevisiae*, disruption of *CRD1*, a gene encoding the yeast CLS, significantly increased PG content in the membrane when grown in a nonfermentable carbon source, but caused PG depletion when grown in medium containing glucose [11]. PG levels in yeast also regulate the expression of the *CRD1* gene. The expression of the *CRD1* gene was repressed by PG deficiency caused by mutation of the yeast *PGS1* gene, whereas the

CRD1 gene expression was significantly elevated in the *crd1* mutant yeast strain that exhibit high level of PG content [20].

We and others have recently cloned and characterized a human gene encoding the first mammalian CL synthase, hCLS1 [21–23]. The identification of hCLS1 provides us an opportunity to investigate how hCLS1 regulates PG synthesis and remodeling. In this report, we investigated a role of hCLS1 in regulating the PG biosynthesis and remodeling in cultured COS-7 cells. The results from the current studies identified a novel function of hCLS1 as a mitochondrial acyl-CoA dependent acyltransferase that catalyzed efficiently the resynthesis of PG from LPG using various acyl-CoAs as substrates both *in vitro* and in cultured COS-7 cells.

MATERIALS AND METHODS

Expression of hCLS1, ALCAT1, and LPGAT1 in COS-7 Cells

COS-7 cells were maintained under the conditions recommended by American Tissue Culture Collection (ATCC, Manassas, VA). One day before transfection, two million cells were sub-cultured onto a 100 × 20 mm plate resulting in ~70% confluence. The COS-7 cells were transfected with 10 µg of the expression vector for hCLS1, ALCAT1, and LPGAT1 respectively or co-transfected with hCLS1 and LPGAT1 expression vectors, depending on the experimental design, using FuGENE 6 (Roche Diagnostics, Indianapolis, IN). Forty eight hours after the transfection, the COS-7 cells were washed once with ice-cold PBS buffer, and pelleted by centrifugation. The cell pellets were homogenized in a lysis buffer that contains 1% Triton X-100 in PBS and 1x Complete protease inhibitors (Roche Diagnostics, Indianapolis, IN). The protein concentration in homogenates was determined by a BCA protein assay kit (Pierce) according to the manufacturer's instructions. The homogenates (50 µg) were then used to assess the CLS, ALCAT, or LPGAT enzyme activity.

Overexpression of the recombinant hCLS1 in Sf-9 insect cells

Spodoptera frugiperda (Sf-9) insect cells were grown in SF-900™ SFM serum free insect medium (Invitrogen). Sf-9 cells were maintained at 28°C at a density between 1×10⁶ and 3×10⁶ cells/mL with shaking at 115 rpm. The flag-hCLS1 cDNA was subcloned from pcDNA3.1 expression vector into the *Bam* HI and *Xho* I sites of pFastBac vector (Invitrogen) and was used for the generation of recombinant baculoviruses overexpressing hCLS1 using the *Bac-to-Bac* baculovirus expression system (Invitrogen). The recombinant baculoviruses were then used to infect Sf-9 insect cells to produce the recombinant hCLS1 protein. Sf-9 cells were typically infected with the recombinant baculoviruses or vector control baculoviruses for 72 hours, harvested in ice-cold phosphate-buffered saline, and homogenized in 20 mM NaCl with 10 up-and-down strokes in a motor-driven Dounce homogenizer (Heidolph) followed by 10 passages through a 27-gauge needle. Cell homogenates were immediately subjected to enzyme assays or frozen at –80°C for later use. The protein concentration in homogenates was determined by a BCA protein assay kit (Pierce) according to the manufacturer's instructions.

Purification of the recombinant hCLS1 overexpressed in Sf-9 cells

Sf-9 cells were harvested after 72 hours post infection with baculoviruses by centrifugation at 1,000 ×g for 5 min at 4°C. The cell pellets were washed with ice-cold PBS and then resuspended in cold homogenization buffer (1.5 mM MgCl₂, 20 mM Tris-Cl, pH 7.5, 500 mM NaCl, 20 % glycerol (v/v), 20 µg/ml aprotinin (Sigma), 2 mM DTT, 1 mM PMSF, 20 µg/ml leupeptin (Sigma), 0.2 mM EDTA), and homogenized in a Wheaton dounce homogenizer with 40 strokes. The homogenate was then centrifuged at 190,000 ×g for 30 min, and the supernatant was diluted with cold dilution buffer (1.5 mM MgCl₂, 0.5 % NP-40 (v/v), 20 mM Tris-Cl, pH 7.5, 20 µg/ml aprotinin (Sigma), 1 mM PMSF, 20 µg/ml leupeptin (Sigma), 2 mM DTT, 10 % glycerol (v/v), and 0.2 mM EDTA). The diluted cell lysates were mixed with equal volume

anti-FLAG M2 affinity resin (Sigma) previously equilibrated with dilution buffer, and incubated at 4 °C for 4 hours. The resin was washed 4 times with cold wash buffer (2 mM DTT, 0.2 mM EDTA, 1 mM PMSF, 0.2 % NP-40 (v/v), 10 % glycerol (v/v), 150 mM NaCl, 1.5 mM MgCl₂, and 20 mM Tris-Cl, pH 7.5). The recombinant hCLS was purified by incubating the resin in 150 µl of cold elution buffer that contain 15 % glycerol (v/v), 0.2 mg/ml flag peptide (Sigma), 100 mM NaCl, 20 mM Tris-Cl, pH 7.5, 2 mM DTT, 1 mM PMSF, 0.2 mM EDTA, 0.5 mg/ml BSA, 0.1 % NP-40 (v/v)) for 10 min followed by centrifugation at 1,000 × *g* for 5 min at 4 °C. The supernatant that contained purified hCLS1 were immediately subjected to enzyme assays or frozen at −80°C for later use. The protein concentration in homogenates was determined by a BCA protein assay kit (Pierce) according to the manufacturer's instructions.

In vitro assays for CL synthase activity

CL synthase activity was assayed as previously described [21] in a 200 µl reaction that contained 50 mM Tris-Cl (pH 8.0), 4.0 mM MgCl₂, 50 µM [¹⁴C] oleoyl-CoA (50 mCi/mmol) (American Radiolabeled Chemical, Inc., St. Louis, MO), 200 µM lysophosphatidylglycerol (1-oleoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)]) and 200 µM CDP-DAG (Avanti Polar Lipids, Inc., Alabaster, AL). The reaction was initiated by addition of 50 µg of cell homogenates from COS-7 cells transiently transfected with hCLS1 expression vector or co-transfected with both hCLS1 and LPGAT1 expression vectors, and incubated for 20 min at room temperature. Lipids were extracted by sequential addition of 2 ml of chloroform:methanol (2:1, v/v) and 0.25 ml of 0.9% KCl. After a vigorous vortexing and a brief centrifugation, aliquots of the organic phase (bottom) were dried under a speed vacuum, and separated by the Linear-K Preadsorbent TLC Plate (Waterman Inc., Clifton, NJ) with chloroform:methanol:water (65:25:4, v/v). After separation, TLC plates were exposed to a Phosphorimager Screen to visualize the radiolabeled products using a Molecular Dynamics STORM 860 Scanner (Sunnyvale, CA). Absolute enzymatic activity (nmol/min per mg protein) was calculated by scraping radiolabeled phospholipids into scintillation vials followed by scintillation counting with a Beckman LS 6500 Scintillation System (Fullerton, CA).

In vitro assays for LPGAT activity

LPG acyltransferase assays were carried out as previously described [12]. Briefly, after determination of initial reaction rates, enzymatic reaction was initiated by addition of 0.1–1.0 ng of purified flag-tagged hCLS protein or 50 µg of cell homogenates that contained the recombinant LPGAT1 or hCLS1 transiently expressed in COS-7 cells or in Sf-9 cells, and incubated for 20 min at room temperature in a 200-µl reaction that contained 80 mM Tris-Cl, pH 7.0, 200 µM 1-oleoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)](sodium salt) (Avanti Polar Lipids, Inc., Alabaster, AL), and 50 µM various [¹⁴C]-labeled oleoyl-CoA (50 mCi/mmol) (American Radiolabeled Chemicals Inc., St. Louis, MO). Lipid extraction, TLC analysis, and quantification of [¹⁴C]-labeled PG was carried out as described above.

In vitro assays for acyl-CoA dependent lysoCL acyltransferase activity

LysoCL acyltransferase assays were carried out as previously described [24]. Acyl-CoA:lysoCL acyltransferase activity was determined by measuring the incorporation of radiolabeled acyl moieties of [¹⁴C] oleoyl-CoA (acyl donor) into monolysoCL in a reaction mixture that contained 50 mM Tris-Cl, pH 7.0, 200 µM monolysoCL, 50 µM [¹⁴C] acyl-CoA (50 mCi/mmol, American Radiolabeled Chemicals Inc), and 50 µg cell lysate from COS-7 cells transiently transfected with the ALCAT1 expression vector or the hCLS1 expression plasmid for 20 min at room temperature. Lipid extraction, TLC analysis, and quantification of [¹⁴C]-labeled CL was carried out as described above.

Analysis of CL synthase and LPG acyltransferase activity of hCLS1 in intact COS-7 cells

COS-7 cells were transfected with 1–4 µg of the hCLS1 expressing vector using FuGENE 6 (Roche Diagnostics, Indianapolis, IN) following the manufacturer's instructions. Twenty four hours after the transfection, the cells were cultured in medium supplemented with 100 µM [¹⁴C] oleoyl-CoA to label newly synthesized lipids during 24 hours. The culture medium was also supplemented with 500 µM oleoyl-LPG to measure LPG acyltransferase activity or 500 µM oleoyl-LPG plus 500 µM CDP-DAG to analyze CL synthase activity of the transfected COS-7 cells. After 24 hours labeling, cells were rinsed twice with ice-cold PBS, and homogenized on ice, followed by lipid extraction, TLC analysis, and quantification of [¹⁴C]-labeled phospholipids as previously described [21].

Statistical analysis

All quantitative data were expressed as mean ± S. D. Statistical analyses for differences between two groups were carried out using Student's *t* test.

RESULTS

Identification of acyl-CoA dependent LPG acyltransferase activity of the recombinant hCLS1

PG is a precursor for CL synthesis, and its biosynthesis has been shown to be coupled with CL synthesis. We have recently cloned and characterized a gene encoding the human cardiolipin synthase (hCLS1), and demonstrated that the hCLS1 catalyzes efficiently the CL synthesis using PG and CDP-DAG as substrates. In the process, the hCLS1 was co-expressed with the human LPGAT1 which was used to synthesize [¹⁴C]-PG as a substrate for the CLS assay. LPGAT1 is an acyltransferase recently identified in this lab, and catalyzes synthesis of PG by acylating lysophosphatidylglycerol (LPG) to PG [12]. Surprisingly, the co-expression of LPGAT1 and hCLS1 resulted in a significant increase in [¹⁴C]-PG production, in addition to the expected synthesis of CL from the enzyme reaction (Fig 1). The increase in LPG acyltransferase activity was not caused by an increase in the expression of the recombinant Flag-tagged LPGAT1 (Fig 1). The results suggest that hCLS1 possess acyl-CoA dependent LPG acyltransferase activity.

To provide direct evidence that hCLS1 catalyzes the acylation of LPG to PG, we next analyzed acyl-CoA dependent LPG acyltransferase activity of the recombinant hCLS1 protein using LPGAT1 as a positive control for the enzymatic reactions. LPGAT1 and hCLS1 were transiently expressed in COS-7 cells, and analyzed for both CL synthase activity and LPG acyltransferase activity with [¹⁴C] oleoyl-CoA, *sn*-1-oleoyl-LPG, and CDP-DAG as substrates. As shown in Figure 2A, the hCLS1 enzyme expressed in COS-7 cells catalyzed efficiently the synthesis of CL only in the presence of CDP-diacylglycerol, [¹⁴C] oleoyl-CoA, and LPG, confirming its functional role in CL synthesis. In contrast, the recombinant LPGAT1 is devoid of CL synthase activity, as supported by the absence of [¹⁴C]-CL from the enzyme assay. Concurrently, the recombinant hCLS1 also displayed strong acyltransferase activity toward LPG, as evidenced by a significant increase in the level of [¹⁴C]-PG when compared with that from the COS-7 cells transfected with an empty expression vector. As a positive control for the LPG acyltransferase assay, the recombinant LPGAT1 protein catalyzed efficiently the acylation of LPG to PG, as previously reported [12]. Furthermore, The LPG acyltransferase activity of the recombinant hCLS1 enzyme is independent from its CL synthase activity. As shown in Figure 2B, the hCLS1 enzyme expressed in COS-7 cells exhibited strong LPG acyltransferase activity in the absence of CDP-DAG which is required for CL synthase activity of the hCLS1 enzyme, as evidenced by a significant increase in [¹⁴C]-PG production when compared with that from the COS-7 cells transfected with the empty expression vector (control). Once again, the LPGAT1 enzyme demonstrated strong LPG acyltransferase activity as expected in the absence of CDP-DAG.

The recombinant hCLS1 protein was deficient in acyl-CoA dependent lysoCL acyltransferase activity

PG and CL are polyglycerolphospholipids, and they share a similar chemical structure more than any other phospholipids. To decipher a role of hCLS1 in CL remodeling, we next analyzed lysoCL acyltransferase activity of the recombinant hCLS1 protein expressed in COS-7 cells using ALCAT1 as a positive control. ALCAT1 is an acyl-CoA:lysoCL acyltransferase recently identified in our lab, and catalyzes the synthesis of CL from lysoCL with acyl-CoA as an acyl donor [24]. As shown in Figure 3, the recombinant ALCAT1 protein catalyzed efficiently the acylation of monolysoCL with acyl-CoA as an acyl donor. In contrast, the recombinant hCLS1 protein was completely inactive towards lysoCL, suggesting the hCLS1 enzyme is devoid of ALCAT enzyme activity. The results suggest that the acyltransferase activity of hCLS1 is specific for LPG.

Analysis of acyl selectivity of hCLS1 towards various acyl-CoAs

As shown by previous studies, the recombinant hCLS1 enzyme lacks a preference to linoleic acid, the dominant acyl composition in CL [22]. Instead, the hCLS1 enzyme demonstrates a slight preference to C18:1 and C18:2 PG when overexpressed in yeast cells [22]. To investigate the acyl selectivity of hCLS1, we analyzed LPG acyltransferase activity of the recombinant hCLS1 enzyme towards various acyl-CoAs that differ in chain length and degree of saturation. As shown in Figure 4, the recombinant hCLS1 exhibited significant LPG acyltransferase activity towards all the acyl-CoAs used in the experiments, and demonstrated an acyl preference to the acyl-CoAs in the order of C18:1 > C18:2 > 18:0 > 16:0. The result is consistent with the acyl selectivity of the hCLS1 enzyme towards PG in CL synthesis, suggesting PG remodeling activity catalyzed by hCLS1 is an intrinsic property of the enzyme. Furthermore, the acyl selectivity of hCLS1 is also similar to that of LPGAT1 [12], further supporting a role of hCLS1 in PG remodeling.

The recombinant hCLS1 enzyme catalyzed efficiently the synthesis of PG and CL in live COS-7 cells

In order to provide further evidence that the hCLS1 enzyme functions as an acyl-CoA dependent LPG acyltransferase, we next analyzed CL and PG biosynthesis in live COS-7 cells transiently transfected with an hCLS1 expression plasmid. The transfected COS-7 cells were cultured in the presence of [¹⁴C] oleoyl-CoA for 24 hours to label the newly synthesized lipids. As demonstrated in Fig 5A, the recombinant hCLS1 enzyme catalyzed efficiently the synthesis of CL in intact COS-7 cells, as evidenced by the increased levels of radiolabeled CL in proportion to the amount of the hCLS1 plasmid used in the transient transfection experiment. Concurrently, the overexpression of hCLS1 in COS-7 cells also resulted in dose-dependent increase in [¹⁴C]-PG, without any significant effects on the biosynthesis of other phospholipids. The synthesis of [¹⁴C]-CL is dependent upon the presence of CDP-DAG and *sn*-1-oleoyl-LPG supplement in the medium. In the absence of CDP-DAG, there is no significant increase in [¹⁴C]-CL in the intact cells (Figure 5B). In contrast, the biosynthesis of [¹⁴C]-PG by hCLS1 did not require the presence of CDP-DAG, further confirming that the LPG acyltransferase activity of hCLS1 enzyme is independent from its CL synthase activity.

The recombinant hCLS1 enzyme purified from Sf-9 insect cells possessed strong LPG acyltransferase activity

In order to provide conclusive evidence that the hCLS1 enzyme functions as an acyl-CoA dependent LPG acyltransferase, we next generated recombinant baculoviruses overexpressing hCLS1 for highly efficient overexpression of flag-tagged hCLS1 in Sf-9 insect cells. As shown by Fig 6, infection of Sf-9 cells with the recombinant baculoviruses resulted in high level of expression of the hCLS1 protein (panel A, lane 3), which was confirmed by Western blot

analysis using anti-flag antibody (lane 3, panel B). The Sf-9 cell infected with the recombinant hCLS1 was used to purify the recombinant hCLS1 protein by anti-flag affinity column. As shown by Fig 6 (lane 4–6), the purification resulted in significant enrichment of hCLS1 protein as detected by commassie blue staining and confirmed by Western blot analysis (panel B, lane 4–6).

We next compared LPG acyltransferase activity of the partially purified hCLS1 with that of cell lysate from either COS-7 cells transiently overexpressing LPGAT1 or from Sf-9 cells overexpressing the hCLS1. Consistent with high expression of hCLS1 in Sf-9 cells, cell lysate from Sf-9 overexpressing hCLS1 demonstrated significantly higher LPG acyltransferase activity (lane 3–4) than those from the COS-7 cells overexpressing LPGAT1 which was used as positive control for the enzyme assay (lane 1–2). Furthermore, the purified hCLS1 possessed strong LPG acyltransferase activity, as confirmed by dose-dependent acyltransferase activity of the purified protein toward LPG using acyl-CoA as an acyl donor (lane 5–8).

DISCUSSION

PG is a precursor for CL biosynthesis and an important signal molecule that regulates various cellular functions. PG is subjected to remodeling subsequent to its *de novo* biosynthesis in mitochondria to incorporate appropriate acyl content for its biological functions and to prevent the harmful effect of LPG accumulation. An acyl-CoA dependent PG remodeling activity has previously been reported from different subcellular fractions, including ER and mitochondria [13]. A gene encoding an ER-associated acyl-CoA dependent LPG acyltransferase (LPGAT1) has recently been identified and characterized by our group [12]. In contrast, little is known about the molecular identity of the LPGAT isoform in mitochondria where PG is synthesized. In this report, we identified a novel function of the hCLS1 enzyme as an acyl-CoA dependent LPG acyltransferase. In support of a regulatory role of hCLS1 in PG remodeling, the recombinant hCLS1 expressed in COS-7 catalyzed efficiently the acylation of LPG to PG using acyl-CoA as an acyl donor. The acyltransferase activity of hCLS1 is specific for LPG, since the recombinant hCLS1 enzyme displayed no acyltransferase activity toward lysoCL which shares a similar molecular structure with LPG. Furthermore, overexpression of hCLS1 in COS-7 cells resulted in a significant increase in PG synthesis concurrent with elevated level of radio-labeled CL in live COS-7 cells without any detectable effects on the biosynthesis of other phospholipids. The LPG acyltransferase activity of the hCLS1 enzyme did not require the presence of CDP-DAG, suggesting that the LPG acyltransferase activity of hCLS1 enzyme is independent from its CL synthase activity. It is possible that the observed LPG acyltransferase activity could be caused by up-regulation of novel isoforms of LPGAT in response to overexpression of hCLS1 in COS-7 cells. However, this possibility has been challenged by our observation that the purified hCLS1 protein from Sf-9 insect cells demonstrated strong LPG acyltransferase activity. Taken together, our results suggest that hCLS1 is a multifunctional enzyme that catalyzes two consecutive steps in CL biosynthesis by first acylating LPG to PG and then transferring a phosphatidyl residue from CDP-diacylglycerol to PG.

The hCLS1 is the first mammalian CLS recently identified by us and others [21–23]. In eukaryotic cells, CLS is involved in the final step of CL synthesis by catalyzing the transfer of a phosphatidyl residue from CDP-diacylglycerol to PG. In yeast *S. cerevisiae*, CLS plays an important role in regulating mitochondrial function. Mutation of the yeast *CRD1* gene encoding a CL synthase results in CL deficiency accompanied by impaired viability, reduced membrane potential, and defective oxidative phosphorylation [7]. CL biosynthesis also plays an important role in regulating mitochondrial function in mammals. CL deficiency in Chinese hamster ovary (CHO) cells caused by mutation of the *PGS1* gene results in morphological and functional mitochondrial abnormalities, manifested by more stringent temperature sensitivity for cell

growth in glucose-deficient medium and by reduced ATP production [25]. The mutant CHO cells demonstrate an increased glycolysis, reduced oxygen consumption, and defective respiratory electron transport chain activity. Furthermore, CL deficiency has recently been implicated a role in mitochondrial dysfunction associated with metabolic diseases, such as diabetes and cardiovascular disease [26–30].

It has long been speculated that CLS, like other metabolic enzymes involved in the synthetic pathway of CL, lacks acyl selectivity towards its substrates. However, this concept has been shown by our findings to be overly simplified. In contrast to a lack of preference to CDP-DAGs [22], our results show that the hCLS1 exhibited clear preference to oleoy- and linoleoy-CoAs as acyl donors when analyzed for its LPG acyltransferase activity. The acyl selectivity profile of hCLS toward acyl-CoAs in PG remodeling is similar to that of hCLS1 toward PGs in CL synthesis [22], suggesting that the PG remodeling by hCLS1 is an intrinsic property of the enzyme to safeguard the acyl composition of PG for the biosynthesis of CL. In support of this hypothesis, the acyl composition of PG has been shown to affect hCLS enzyme activity [22]. Furthermore, the acyl selectivity profile of hCLS1 is similar to that of the mitochondrial isoform of LPGAT previously reported in rat heart [13], suggesting that hCLS1 may represent the same enzyme since hCLS1 is exclusively localized in mitochondria [21].

Cumulative evidence suggests that PG remodeling is coupled with the biosynthesis of CL by CLS. Yet, the underlying molecular mechanism remains elusive. In rat hearts perfused with [¹⁴C]-glycerol, there was a time-dependent accumulation of radioactivity incorporated into CL and LPG, but not other phospholipids involved in the biosynthesis of CL [14], suggesting that CL synthesis requires an active remodeling of PG. In support of the observation, an increase in CLS activity is associated with elevated levels of PG remodeling in cultured lung cell line [31]. Furthermore, LPG has been shown to inhibit CLS activity from various organisms [15]. Defective CL remodeling caused by Barth syndrome is associated with linoleic acid deficiency in both CL and PG, but not other phospholipids [18]. Both CL and PG are substantially depleted by the onset of diabetes [28]. In *S. cerevisiae*, disruption of *CRD1*, a gene encoding the yeast CL synthase, caused PG depletion when grown in medium containing glucose [11]. Therefore, our work on identification of hCLS1 as a novel mitochondrial LPG acyltransferase offers a possible molecular mechanism underlying the reported effects of CLS on PG synthesis and remodeling.

Although the mammalian CLS was purified more than two decades ago [32], this is the first time a novel function has been assigned to CLS. Therefore, our findings on hCLS1 as a novel PG remodeling enzyme have important implications. PG is a precursor for the biosynthesis of both CL and lysobisphosphatidic acid (LBPA). The biological significance of PG remodeling by CLS1 is underscored by the previous observations that defective PG remodeling is associated with linoleic acid deficiency in CL and decreased CL levels in Barth syndrome [18]. Furthermore, an increased in saturated fatty acid content in PG has been shown to inhibit CLS enzyme activity and induce cardiomyocyte apoptosis by triggering cytochrome c release [19]. LBPA is a phospholipid initially identified in macrophages, and regulates the transfer of cholesterol to membranes [33] as well as the structure and function of Golgi network [34]. Defective synthesis and remodeling of LBPA is associated with the onset of neuronal and lipid storage diseases [35–37]. However, the enzymes that catalyze the synthesis and remodeling of LBPA remain elusive. Consequently, our work provides an important guidance for future studies to identify additional biological functions of hCLS1 beyond its current roles in regulating CL synthesis and mitochondrial functions.

Acknowledgments

This study was supported in part by grants from NIH (DK076685, Y.S.) and American Diabetes Association (ADA, 7-07-RA-148, Y.S.).

The abbreviations used are

CL	cardiolipin
hCLS	human cardiolipin synthase
PG	phosphatidylglycerol
LPG	lysophosphatidylglycerol
lysoCL	lysocardiolipin
CDP-DAG	CDP-diacylglycerol
PGS	phosphatidylglycerophosphate synthase
LBPA	lysobisphosphatidic acid
PBS	phosphate-buffered saline
ER	endoplasmic reticulum

References

1. Mileykovskaya E, Zhang M, Dowhan W. Cardiolipin in energy transducing membranes. *Biochemistry-Russia* 2005;70:154–8.
2. Li G, Chen S, Thompson MN, Greenberg ML. New insights into the regulation of cardiolipin biosynthesis in yeast: Implications for Barth syndrome. *Biochim Biophys Acta* 2007;1771:432–41. [PubMed: 16904369]
3. Paradies G, Petrosillo G, Pistolese M, Di Venosa N, Federici A, Ruggiero FM. Decrease in mitochondrial complex I activity in ischemic/reperfused rat heart: involvement of reactive oxygen species and cardiolipin. *Circul Res* 2004;94:53–9.
4. Lutter M, Fang M, Luo X, Nishijima M, Xie X, Wang X. Cardiolipin provides specificity for targeting of tBid to mitochondria. *Nat Cell Biol* 2000;2:754–61. [PubMed: 11025668]
5. McMillin JB, Dowhan W. Cardiolipin and apoptosis. *Biochim Biophys Acta* 2002;1585:97–107. [PubMed: 12531542]
6. Kagan VE, Tyurin VA, Jiang J, Tyurina YY, Ritov VB, Amoscato AA, Osipov AN, Belikova NA, Kapralov AA, Kini V, Vlasova II, Zhao Q, Zou M, Di P, Svistunenko DA, Kurnikov IV, Borisenko GG. Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nat Chem Biol* 2005;1:223–32. [PubMed: 16408039]
7. Zhong Q, Gohil VM, Ma L, Greenberg ML. Absence of cardiolipin results in temperature sensitivity, respiratory defects, and mitochondrial DNA instability independent of pet56. *J Biol Chem* 2004;279:32294–300. [PubMed: 15169766]
8. Schlame M, Rua D, Greenberg ML. The biosynthesis and functional role of cardiolipin. *Progress in Lipid Research* 2000;39:257–88. [PubMed: 10799718]
9. Chicco AJ, Sparagna GC. Role of cardiolipin alterations in mitochondrial dysfunction and disease. *Am J Physiol Cell Physiol* 2007;292:C33–44. [PubMed: 16899548]
10. Kawasaki K, Kuge O, Chang SC, Heacock PN, Rho M, Suzuki K, Nishijima M, Dowhan W. Isolation of a chinese hamster ovary (CHO) cDNA encoding phosphatidylglycerophosphate (PGP) synthase, expression of which corrects the mitochondrial abnormalities of a PGP synthase-defective mutant of CHO-K1 cells. *J Biol Chem* 1999;274:1828–34. [PubMed: 9880566]
11. Jiang F, Ryan MT, Schlame M, Zhao M, Gu Z, Klingenberg M, Pfanner N, Greenberg ML. Absence of cardiolipin in the crd1 null mutant results in decreased mitochondrial membrane potential and reduced mitochondrial function. *J Biol Chem* 2000;275:22387–94. [PubMed: 10777514]

12. Yang Y, Cao J, Shi Y. Identification and characterization of a gene encoding human LPGAT1, an endoplasmic reticulum-associated lysophosphatidylglycerol acyltransferase. *J Biol Chem* 2004;279:55866–74. [PubMed: 15485873]
13. Cheng P, Dolinsky V, Hatch GM. The acylation of lysophosphatidylglycerol in rat heart: evidence for both in vitro and in vivo activities. *Biochim Biophys Acta* 1996;1302:61–8. [PubMed: 8695656]
14. Cao SG, Hatch GM. Cardiolipin is synthesized from exogenous phosphatidylglycerol in rat heart. *Biochim Biophys Acta* 1995;1256:97–102. [PubMed: 7742361]
15. Schlame M, Brody S, Hostetler KY. Mitochondrial cardiolipin in diverse eukaryotes. Comparison of biosynthetic reactions and molecular acyl species. *Eur J Biochem* 1993;212:727–35. [PubMed: 8385010]
16. Neuwald AF. Barth syndrome may be due to an acyltransferase deficiency. *Curr Biol* 1997;7:R465–6. [PubMed: 9259571]
17. Xu Y, Malhotra A, Ren M, Schlame M. The enzymatic function of tafazzin. *J Biol Chem* 2006;281:39217–24. [PubMed: 17082194]
18. Vreken P, Valianpour F, Nijtmans LG, Grivell LA, Plecko B, Wanders RJ, Barth PG. Defective remodeling of cardiolipin and phosphatidylglycerol in Barth syndrome. *Biochem Biophys Res Commun* 2000;279:378–82. [PubMed: 11118295]
19. Ostrander DB, Sparagna GC, Amoscato AA, McMillin JB, Dowhan W. Decreased cardiolipin synthesis corresponds with cytochrome c release in palmitate-induced cardiomyocyte apoptosis. *J Biol Chem* 2001;276:38061–7. [PubMed: 11500520]
20. Su X, Dowhan W. Regulation of cardiolipin synthase levels in *Saccharomyces cerevisiae*. *Yeast* 2006;23:279–91. [PubMed: 16544270]
21. Chen D, Zhang XY, Shi Y. Identification and functional characterization of hCLS1, a human cardiolipin synthase localized in mitochondria. *Biochem J* 2006;398:169–76. [PubMed: 16716149]
22. Houtkooper RH, Akbari H, van Lenthe H, Kulik W, Wanders RJA, Frentzen M, Vaz FM. Identification and characterization of human cardiolipin synthase. *FEBS Lett* 2006;580:3059–64. [PubMed: 16678169]
23. Lu B, Xu FY, Jiang YJ, Choy PC, Hatch GM, Grunfeld C, Feingold KR. Cloning and characterization of a cDNA encoding human cardiolipin synthase (hCLS1). *J Lipid Res* 2006;47:1140–5. [PubMed: 16547353]
24. Cao J, Liu Y, Lockwood J, Burn P, Shi Y. A novel cardiolipin-remodeling pathway revealed by a gene encoding an endoplasmic reticulum-associated acyl-CoA:lysocardiolipin acyltransferase (ALCAT1) in mouse. *J Biol Chem* 2004;279:31727–34. [PubMed: 15152008]
25. Ohtsuka T, Nishijima M, Suzuki K, Akamatsu Y. Mitochondrial dysfunction of a cultured Chinese hamster ovary cell mutant deficient in cardiolipin. *J Biol Chem* 1993;268:22914–9. [PubMed: 8226801]
26. Petrosillo G, Ruggiero FM, Di Venosa N, Paradies G. Decreased complex III activity in mitochondria isolated from rat heart subjected to ischemia and reperfusion: role of reactive oxygen species and cardiolipin. *FASEB J* 2003;17:714–6. [PubMed: 12586737]
27. Lesnefsky EJ, Chen Q, Slabe TJ, Stoll MS, Minkler PE, Hassan MO, Tandler B, Hoppel CL. Ischemia, rather than reperfusion, inhibits respiration through cytochrome oxidase in the isolated, perfused rabbit heart: role of cardiolipin. *American J Physiol - Heart & Cir Physiol* 2004;287:H258–67.
28. Han X, Yang J, Cheng H, Yang K, Abendschein DR, Gross RW. Shotgun lipidomics identifies cardiolipin depletion in diabetic myocardium linking altered substrate utilization with mitochondrial dysfunction. *Biochemistry* 2005;44:16684–94. [PubMed: 16342958]
29. Watkins SM, Reifsnyder PR, Pan HJ, German JB, Leiter EH. Lipid metabolome-wide effects of the PPARgamma agonist rosiglitazone. *J Lipid Res* 2002;43:1809–17. [PubMed: 12401879]
30. Pan HJ, Lin Y, Chen YE, Vance DE, Leiter EH. Adverse hepatic and cardiac responses to rosiglitazone in a new mouse model of type 2 diabetes: relation to dysregulated phosphatidylcholine metabolism. *Vascul Pharmacol* 2006;45:65–71. [PubMed: 16750656]
31. Rusnak A, Mangat R, Xu F, McClarty G, Hatch GM. Cardiolipin remodeling in a Chinese hamster lung fibroblast cell line deficient in oxidative energy production. *J Bioenerg & Biomemb* 1997;29:291–8.

32. McMurray WC, Jarvis EC. Partial purification of diphosphatidylglycerol synthetase from liver mitochondrial membranes. *Can J Biochem* 1980;58:771–6. [PubMed: 6257342]
33. Kobayashi T, Beuchat MH, Lindsay M, Frias S, Palmiter RD, Sakuraba H, Parton RG, Gruenberg J. Late endosomal membranes rich in lysobisphosphatidic acid regulate cholesterol transport.[see comment]. *Nat Cell Biol* 1999;1:113–8. [PubMed: 10559883]
34. Cluett EB, Kuismanen E, Machamer CE. Heterogeneous distribution of the unusual phospholipid semilyso-bisphosphatidic acid through the Golgi complex. *Mol Biol Cell* 1997;8:2233–40. [PubMed: 9362065]
35. Salvioli R, Scarpa S, Ciaffoni F, Tatti M, Ramoni C, Vanier MT, Vaccaro AM. Glucosylceramidase mass and subcellular localization are modulated by cholesterol in Niemann-Pick disease type C. *J Biol Chem* 2004;279:17674–80. [PubMed: 14757764]
36. Kakela R, Somerharju P, Tyynela J. Analysis of phospholipid molecular species in brains from patients with infantile and juvenile neuronal-ceroid lipofuscinosis using liquid chromatography-electrospray ionization mass spectrometry. *J Neurochem* 2003;84:1051–65. [PubMed: 12603829]
37. Fobker M, Voss R, Reinecke H, Crone C, Assmann G, Walter M. Accumulation of cardiolipin and lysocardiolipin in fibroblasts from Tangier disease subjects. *FEBS Lett* 2001;500:157–62. [PubMed: 11445077]

Figure 1. Co-expression of hCLS1 and LPGAT1 in COS-7 cells increases LPG acyltransferase activity

The COS-7 cells were transiently transfected with pcDNA3.1 vector (control), the LPGAT1 expression plasmid, or co-transfected with both LPGAT1 and hCLS1 expression vectors. Forty eight hours after the transfection, the cells were homogenized in cell lysis buffer, and the cell lysate (50 μg) was used for the cardiolipin synthase assay. Cardiolipin synthase activity was measured in a 200 μl enzyme reaction that contained 50 mM Tris-Cl (pH 8.0), 4.0 mM MgCl_2 , 20 μM $[^{14}\text{C}]$ oleoyl-CoA, 200 μM lysophosphatidylglycerol (LPG) and 200 μM CDP-diacylglycerol (CDP-DAG). The reactions were terminated, and lipids were extracted with chloroform/methanol (2:1, v/v), followed by TLC and PhosphorImager analyses. The levels

of [^{14}C]-phosphatidylglycerol were quantified and shown in the lower panel. The position of the [^{14}C]-labeled phosphatidylglycerol (PG), cardiolipin (CL), and free fatty acid (FFA) on a TLC plate was indicated by an arrow. The protein level of Flag-tagged LPGAT1 used in the enzyme reactions were analyzed by Western blot analysis using anti-Flag antibody (bottom)

Figure 2A

Figure 2B

Figure 2. The recombinant hCLS1 enzyme exhibits a strong acyl-CoA dependent LPG acyltransferase activity

The COS-7 cells were transiently transfected with expression vectors for LPGAT1, hCLS1, or the pcDNA3.1 vector (control). Forty eight hours after transfection, the COS-7 cells were homogenized in cell lysis buffer. 50 μg of the cell lysate was used to measure the LPG acyltransferase and CL synthase activity using the same assay conditions as described in figure 1 in the presence (A) or absence (B) of 200 μM CDP-DAG. A representative TLC analysis of the radio-labeled CL and PG is shown in the top panel. The data from three independent experiments were quantified and expressed as mean $\pm$ S.D in the lower panel. ** $p < 0.01$ when

compared with the empty vector control. The position of [^{14}C]-labeled phosphatidylglycerol (PG), cardiolipin (CL), and free fatty acid (FFA) on a TLC plate was indicated by an arrow.

Figure 3. The recombinant hCLS1 enzyme does not recognize lysocardiolipin as a substrate
 COS-7 cells were transiently transfected with expression vectors for ALCAT1, hCLS1 or pcDNA3.1 vector (control). Forty eight hours after transfection, the COS-7 cells were homogenized in cell lysis buffer and 50 μg of the cell lysate were used to measure lysoCL acyltransferase activity in a 200 μl of enzyme reaction that contained 50 μM $[^{14}\text{C}]$ oleoyl-CoA and 200 μM of monolysocardiolipin (monolysoCL) for 20 min at room temperature. A representative TLC analysis of the radio-labeled CL is shown in the top panel. The data from three independent experiments were quantified and expressed as mean $\pm$ S.D. in the lower panel. ** $p < 0.01$ when compared with the empty vector control.

Figure 4. Analysis of acyl selectivity profile of the recombinant hCLS1 towards acyl-CoAs
 The recombinant hCLS1 enzyme expressed in COS-7 cells were analyzed for the LPG acyltransferase activity in enzyme reactions that contained 50 mM Tris-Cl (pH 8.0), 4.0 mM MgCl₂, 200 μ M LPG, and 50 μ M of various [¹⁴C] oleoyl-CoAs, including palmitoyl-CoA (C16:0), stearoyl-CoA (C18:0), oleoyl-CoA (18:1), and linoleoyl-CoA (18:2) in the presence of 50 μ g of cell homogenates from COS-7 cells transfected with the hCLS1 expression vector or vector control (Con), followed by lipids extraction and TLC analysis. All enzyme activity data were derived from at least three independent experiments and were shown as mean $\pm$ S.D. **p<0.01 when compared with the vector control.

Figure 5A

Figure 5B

Figure 5. Stimulation of CL and PG biosynthesis by the recombinant hCLS1 in intact COS-7 cells
COS-7 cells were transiently transfected with an increasing amount of hCLS1 expression plasmid (0.0 to 4 μg), and cultured in a medium supplemented with 100 μM [^{14}C] oleoyl CoA to label newly synthesized lipids during 24 hours. The culture medium was also supplemented with 500 μM oleoy-LPG in the presence (A) or absence (B) of 500 μM CDP-DAG to analyze the CLS activity and LPG acyltransferase activity of the transfected COS-7 cells. Total lipids were extracted and subjected to TLC analysis as described above. Results from three independent experiments were quantified and expressed as mean $\pm$ S.D. in the lower panel. The position of the [^{14}C]-labeled phosphatidylglycerol (PG), cardiolipin (CL) and free fatty

acid (FFA) on a TLC plate was indicated by an arrow. All enzyme activity data were derived from at least three independent experiments and were shown as mean $\pm$ S.D.

Figure 6. Analysis of the purified recombinant hCLS1 overexpressed in Sf-9 insect cells by coomassie staining and Western blot analysis

A, coomassie blue staining of the purified recombinant hCLS1 and protein lysates of Sf-9 insect cells resolved on 10% SDS-PAGE. *Lane 1*, protein lysate from Sf-9 cells infected with the vector control baculoviruses. *Lane 2*, uninfected Sf-9 cell extract. *Lane 3*, protein lysate from Sf-9 cells infected with recombinant baculoviruses overexpressing flag-tagged hCLS1. *Lane 4–6*, 1.0ng, 0.3ng, and 0.1ng of purified recombinant flag-tagged hCLS1 protein. *M*, molecular weight marker, kDa. **B**, Western blot analysis of flag-tagged hCLS1 protein from panel A using anti-flag antibody.

Figure 7. Analysis of LPG acyltransferase activity of the purified recombinant hCLS1 from Sf-9 cells

LPG acyltransferase activity was analyzed from 50 μ g of cell lysates prepared from COS-7 cells overexpressing LPGAT1 (lane 1) or vector control (lane 2), Sf-9 cells infected with the recombinant baculoviruses overexpressing hCLS1 (lane 3) or vector control baculoviruses (lane 4), or indicated amount of the purified hCLS1 protein from Sf-9 cells (lane 5–8), respectively, in the presence of 20 μ M [14 C] oleoyl-CoA and 200 μ M LPG for 15 min at room temperature. The radiolabeled lipid products were separated by TLC and analyzed by PhosphorImager analyses.