Comparative Gene Identification 58 (CGI-58)/Alpha Beta Hydrolase Domain 5 (ABHD5) Lacks Lysophosphatidic Acid Acyltransferase Activity

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Running footnote: CGI-58 Lacks LPAAT Activity
Abstract

Mutations in the gene encoding CGI-58/ABHD5 cause Chanarin-Dorfman Syndrome, characterized by excessive triacylglycerol storage in cells and tissues. CGI-58 has been identified as a co-activator of adipose triglyceride lipase (ATGL) and a lysophosphatidic acid acyltransferase (LPAAT). We developed a molecular model of CGI-58 structure, and then mutated predicted active site residues and performed LPAAT activity assays of recombinant wild-type and mutated CGI-58. When mutations of predicted catalytic residues failed to reduce LPAAT activity, we determined that LPAAT activity was due to a bacterial contaminant of affinity purification procedures, plsC, the sole LPAAT in *E. coli*. Purification protocols were optimized to reduce plsC contamination, in turn, reducing LPAAT activity. When CGI-58 was expressed in SM2-1(DE3) cells that lack plsC, lysates lacked LPAAT activity. Additionally, mouse CGI-58 expressed in bacteria as a GST-fusion protein and human CGI-58 expressed in yeast lacked LPAAT activity. Previously reported lipid binding activity of CGI-58 was revisited using protein-lipid overlays. Recombinant CGI-58 failed to bind lysophosphatidic acid, but interestingly, bound phosphatidylinositol 3-phosphate [PI(3)P] and phosphatidylinositol 5-phosphate [PI(5)P]. Pre-binding CGI-58 with [PI(3)P] or [PI(5)P] did not alter its co-activation of ATGL in vitro. In summary, purified recombinant CGI-58 that is functional as an ATGL co-activator lacks LPAAT activity.

**Supplementary key words:** lysophosphatidic acid acyltransferase, phosphatidylinositol 3-phosphate, phosphatidylinositol 5-phosphate, adipose triglyceride lipase, Chanarin-Dorfman Syndrome, neutral lipid storage disorder
Introduction

Mutations in the gene encoding Comparative Gene Identification-58 (CGI-58; also α/β Hydrolase Domain 5, ABHD5) cause Chanarin-Dorfman Syndrome (CDS)(1), a neutral lipid storage disorder characterized by excessive accumulation of triacylglycerols (TAGs) in cells and tissues, including liver, skeletal muscle, intestinal epithelia, leukocytes, keratinocytes and skin fibroblasts, leading to hepatomegaly, ichthyosis, and mild muscle weakness (2,3,4,5,6,7). These early observations suggested that CGI-58 plays an important role in TAG homeostasis in multiple tissues. Although CGI-58 is a member of the lipase subfamily of α/β hydrolase domain proteins, it lacks a serine residue in a conserved sequence (GXSXG) that normally harbors the nucleophilic component of the catalytic triad (1). Identification of CGI-58 as a co-activator of the widely expressed adipose triglyceride lipase (ATGL) (8) provided a mechanistic explanation for the phenotype of CDS patients; loss of functional CGI-58 reduces ATGL-mediated hydrolysis of TAGs, in turn increasing TAG storage in tissues. Individuals with mutations in ATGL show similarities to CDS patients with TAG accumulation in various tissues including liver and skeletal muscle (9); however, these individuals lack ichthyosis, and have more severe skeletal muscle myopathy than individuals with CDS, as well as cardiomyopathy. The phenotypic differences between individuals with mutations in CGI-58 and those with mutations in ATGL suggest that CGI-58 serves one or more functions distinct from co-activation of ATGL.

In 2008, Ghosh and co-workers reported lysophosphatidic acid acyltransferase (LPAAT) activity of recombinant CGI-58 in assays conducted in vitro (10); we later confirmed this activity for recombinant CGI-58 (11). Since phosphatidic acid, the product of the LPAAT reaction, is a potent signaling lipid, these observations suggested that CGI-58 may function in signaling pathways, which could then explain experimental findings of increased insulin sensitivity in livers of mice following CGI-58 knockdown despite significant hepatic steatosis accompanied by elevated levels of diacylglycerol and ceramide (12,13). However, our subsequent studies of CGI-58 enzyme activity have failed to support the original findings of LPAAT activity.
We generated a molecular model for CGI-58 structure and used the model, as well as amino acid homology between CGI-58 of various species, to predict potential catalytic residues responsible for LPAAT activity. Potential catalytic acidic and basic residues of CGI-58 were mutated to alanine residues; mutated forms of recombinant CGI-58 were purified from *E. coli* lysates for use in LPAAT activity assays. All mutated variants of CGI-58 displayed LPAAT activity. LPAAT activity was then shown to be due to co-purification of plsC, the sole bacterial LPAAT, over cobalt affinity resin. Lipid binding activity of CGI-58 was revisited using highly purified recombinant protein; previously reported binding of lyso(phosphatidic acid was not observed, but novel binding activity towards phosphatidylinositol 3-phosphate [PI(3)P] and phosphatidylinositol 5-phosphate [PI(5)P] was identified.
Materials and Methods

Materials

Bacterial expression of mouse CGI-58 cDNA as a 12-histidine (His) fusion protein using the pET-28a vector (Novagen) was previously reported (11). Expression of mouse CGI-58 and β-galactosidase using adenoviral expression vectors was also previously reported (14). The cDNA for mouse ATGL (IMAGE: 30024535) was purchased from the American Type Culture Collection (Manassas, VA). SM2-1 E. coli were obtained from The Coli Genetic Stock Center at Yale University. DH10Bac E. coli were purchased from Invitrogen. BL21(DE3) E. coli were purchased from Novagen. A λDE3 lysogenization kit was purchased from Novagen. Skin fibroblasts from a human with CDS were generously donated by Dr. Rosalind A. Coleman (University of North Carolina-Chapel Hill). WS1 human skin fibroblasts and Sf9 insect cells were purchased from American Type Culture Collection. The yeast strain ict1Δ (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ict1Δ::kanMX) lacking a soluble LPAAT was purchased from Invitrogen. Yeast nitrogen base, raffinose, and galactose were purchased from Sigma.

TALON His-Tag Purification Resin was purchased from Clontech Laboratories, Inc. Glutathione Sepharose 4B was purchased from GE Healthcare Life Sciences. DMEM and MEM were purchased from Mediatech. Bovine serum albumin, fatty acid free bovine serum albumin, and fetal bovine serum were purchased from Sigma. Lipofectamine, Plus Reagent, Cellfectin, and Grace’s Medium were purchased from Invitrogen. Coomassie Plus “The Better Bradford Assay Reagent” and GelCode Blue Stain Reagent were purchased from Pierce.

Rabbit polyclonal antisera raised against full length recombinant mouse CGI-58 was previously described (14). Anti-6-His antibody was purchased from Clontech Laboratories, Inc. Anti-glutathione-S-transferase (GST) antibody was purchased from GE Healthcare. Anti-human ABHD5 antibody was purchased from Fitzgerald Industries. Anti-β-galactosidase, anti-calnexin, and peroxidase-conjugated anti-mouse, anti-rabbit, and anti-goat antibodies were purchased.
from Sigma. Enhanced chemiluminescence reagents were purchased from Pierce.

Phosphatidylinositol phosphate (PIP) strips, PIP Arrays, Membrane Lipid Strips, Sphingo Strips, phosphatidylinositol dIc8, phosphatidylinositol 3-phosphate [PI(3)P], PI(5)P, and PI(4,5)P were purchased from Echelon, Inc. (Salt Lake City, UT). Phosphatidic acid and lysophosphatidic acid were purchased from Sigma. Egg phosphatidylcholine and soy phosphatidylinositol were purchased from Avanti Polar Lipids. Radiolabeled [9,10(N)3H]triolein and [oleoyl-1-14C]oleoyl-CoA were purchased from PerkinElmer Life Sciences (Boston, MA). Hydrofluor scintillation cocktail was purchased from National Diagnostics and ScintiVerse BD Cocktail was purchased from Fisher Scientific. Silica gel hard layer fluorescent TLC plates were obtained from Analtech (Newark, DE). Additional chemicals and organic solvents were purchased from either Fisher Scientific or Sigma.

**Generation of a 3D protein model of CGI-58**

The primary amino acid sequence for mouse CGI-58 (NP_080455.1) was submitted to the online Protein Homology/analogy Recognition Engine (PHYRE) version 0.2 (15). Resulting models were then evaluated using the Structural Analysis and Verification Server (SAVES) (http://nihserver.mbi.ucla.edu/SAVES). The SAVES program includes five programs, including PROCHECK (16), WHAT_CHECK (17), ERRAT (18), VERIFY 3D (19), and PROVE (20), which assess the structural stability of the computer-generated models. The highest ranked model was based on a putative hydrolase (2632844) from *Bacillus subtilis* (Protein Data Bank ID: 2R11). Images of the model were captured with PyMOL software (The PyMOL Molecular Graphics System, Version 1.5.0.1 Schrödinger, LLC).

**Generation of SM2-1(DE3) E. coli**

SM2-1(DE3) E. coli were produced using a λDE3 lysogenization kit to integrate the λDE3 prophage into the SM2-1 E. coli chromosome for isopropyl-β-D-1-thiogalactopyranoside (IPTG)-
induced expression of T7 RNA polymerase, needed for expression of 12-His-tagged CGI-58 using the pET-28a vector. T7 polymerase was detected by immunoblotting bacterial lysates (not shown).

Expression and partial purification of recombinant 12-His-tagged CGI-58

CGI-58 cDNA in a pET-28a vector was used to transform BL21(DE3) or SM2-1(DE3) E. coli; transformants were grown in Luria Broth with kanamycin at 37°C with shaking at 225 rpm to an optical density at 600 nm of 0.6-0.8 before addition of 1 mM IPTG for 5 or 3 hours, respectively. Cells were centrifuged at 4,000 x g for 10 minutes at 4°C, the supernatant was removed, and cell pellets were stored at -20°C.

Thawed cells were suspended in 1 mg/ml lysozyme in lysis buffer containing 50 mM sodium phosphate, pH 7.5, 100 mM potassium chloride, 20 mM imidazole, 1 mM dithiothreitol, and protease inhibitors (Roche Complete, EDTA free), followed by incubation on ice for 30 minutes. Cells were disrupted with glass beads in a Bead-Beater® (Biospec Products, Inc.) chamber surrounded by ice water; cells were disrupted with 10 cycles of 15 seconds blending with 2 minutes intermittent cooling. Lysed cells were centrifuged at 21,000 x g for 20 minutes at 4°C. Clarified supernatant was incubated with 0.5 ml TALON His-Tag Purification Resin for 1.5 hours at 4°C prior to brief centrifugation at 800 x g at 4°C. The resin pellet was resuspended in lysis buffer with either 20 mM imidazole (early experiments) or 100 mM imidazole (later experiments) and transferred to a column for washing with an excess (at least 50 column volumes) of lysis buffer containing either 20 mM imidazole (early experiments) or 100 mM imidazole (later experiments), followed by elution with 250 mM imidazole, 40% glycerol (v/v) in lysis buffer, pH 8.0. Collected fractions were stored at -20°C.

Expression and partial purification of GST-tagged CGI-58

Mouse CGI-58 cDNA was subcloned into the pGEX-4T-1 expression plasmid (GE
Healthcare) to append the nucleotide sequence encoding a GST tag to the 5’ end of the CGI-58 cDNA. Bl21(DE3) E. coli expressing GST-CGI-58 were grown in Luria Broth with shaking at 225 rpm at 37°C to an optical density at 600 nm of 0.6-0.8 before addition of 0.01 mM IPTG for 1-2 hours. Cells were centrifuged at 4,000 x g for 10 minutes at 4°C, the supernatant removed and pelleted cells lysed in PBS with 0.5 mg/ml lysozyme and protease inhibitors. A fraction of lysate was centrifuged at 16,000 x g to remove membranes. Supernatants and whole cell lysates were assayed for LPAAT activity.

**Expression of 6-His plsC**

6-His plsC was expressed in SM2-1(DE3) cells using the same conditions as described for the expression of 12-His-CGI-58 in SM2-1(DE3) cells.

**Expression of ATGL in Sf9 insect cells**

Mouse ATGL cDNA was amplified by PCR using forward (5’-GCCACCATGTTCGAGGAGCAG-3’) and reverse (5’-TTAGTGATGGTGATGGTGATGTCCGCAAGGCGGGAG-3’) primers to add DNA encoding a SpeI restriction site and a Kozak sequence before the start of the coding sequence of ATGL cDNA and a glycine-linked 6-His tag and XhoI restriction site to the 3’ end. The PCR product was ligated into pCR-Blunt (Invitrogen) followed by restriction digestion and ligation into the pFastBac1 vector (Invitrogen). Using the Bac-to-Bac Baculovirus Expression System (Invitrogen), the ATGL-6-His cDNA in pFastBac1 was recombined into a bacmid in DH10Bac E. coli. Recombined bacmids were identified by blue/white screening of colonies, isolated by plasmid mini-preps and confirmed by PCR using M13 forward and reverse primers (Invitrogen). TOP10 E. coli were transformed with bacmids for plasmid purification using the Wizard Plus Midiprep kit (Promega). Cultured Sf9 insect cells were transfected with bacmid complexed with Cellfectin Reagent (Invitrogen) for assembly and propagation of baculovirus. Sf9 cells were maintained in Sf-900II SFM media (Invitrogen) at 28°C. Media were collected 72
hours after transfection and centrifuged at 800 x g for 10 minutes at 4°C to remove cells and debris; supernatants containing baculovirus were stored at 4°C.

For expression of recombinant 6-His-tagged ATGL, Sf9 cells were grown to 70% confluence in 150 mm dishes; growth medium containing 200 µl baculovirus was added for 72 hours to induce protein expression. Media were removed and cell monolayers washed with PBS. Cells were detached from culture dishes by scraping into PBS followed by centrifugation at 800 x g for 10 minutes at 4°C. Supernatants were removed by aspiration and pellets were stored at -70°C.

To prepare cell lysates containing ATGL for TAG hydrolysis assays, frozen cell samples were thawed and resuspended in Sf9 lysis buffer containing 20 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM DTT, with protease inhibitors for 10 minutes on ice. The resuspended cells were disrupted by passing them through a 27.5 gauge syringe 10 times. Lysates were centrifuged at 800 x g for 10 min at 4°C to remove intact cells and heavy membranes.

Expression of human CGI-58 in yeast

To overexpress human CGI-58 with an N-terminal fusion of Protein A in yeast, its cDNA was amplified by PCR using forward (5' GCCCATATGCGGCGGAGGAGGAG 3') and reverse (5' CCGCTCGAGTCAGTCCACAGTGTC 3') primers and was subcloned into the NdeI and XhoI restriction sites of the pYES2 vector (Invitrogen) containing the Protein A sequence derived from PtA-DGK1 (21). The internal NdeI restriction site of the hCGI-58 cDNA was removed by introduction of a silent mutation using the QuikChange® Site-Directed Mutagenesis kit (Stratagene).

Yeast strains BY4741 (22) and ict1Δ (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ict1Δ::kanMX) containing pYES2-PtA-hCGI-58 (or the pYES2 control vector) were grown at 30°C in SC-Ura media containing 2% raffinose as a carbon source. The cultures at the
exponential phase were supplemented with 2% galactose (final concentration) and incubated for 7 hours to express the Protein A-hCGI-58 fusion protein.

Yeast cells expressing hCGI-58 were harvested and suspended in cold lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM potassium chloride, 5 mM magnesium chloride) supplemented with complete EDTA-free protease inhibitors (Roche) and were disrupted using a Mini- Bead-Beater®-16 with 15 pulse sequences of 15 seconds with intermittent cooling. Cell lysates were centrifuged for 10 min at 1500 x g at 4°C to remove unbroken cells and cell debris. The cell extracts were incubated for 30 min with 0.1% (final concentration) Tween-20 at 4°C with gentle rotation, and then centrifuged for 1 hour at 100,000 x g at 4°C in a SW60Ti rotor in a Beckman Ultracentrifuge. The supernatants were collected, 20% (final concentration) glycerol was added, and samples were stored at -20°C.

**Assay for LPAAT Activity**

LPAAT activity of recombinant CGI-58 was assessed by mixing partially purified recombinant CGI-58 (from *E. coli*) or yeast cell extracts with 50 µM lysophosphatidic acid (LPA) and 10 µM [1-14C-oleoyl]oleoyl-CoA in 50 mM Tris-HCl, pH 7.5 (10), followed by incubation of samples for 10 min at 37°C. Reactions were terminated and products extracted by addition of 0.1N HCl in methanol:chloroform:MgCl₂ [1:2:3 (v:v:v)] and centrifugation at 800 x g for 10 minutes at room temperature. After phase separation, the top aqueous phase was discarded and the lower organic phase was dried via speed vacuum centrifugation. The dried organic phase was dissolved in chloroform and spotted onto a silica gel hard layer fluorescent TLC plate. Lipids were separated using developing solvent of chloroform:methanol:acetone:acetic acid:water [50:10:20:15:5 (v:v:v:v:v)], and radiolabeled lipids were visualized using a Storm System Phosphorimager (Molecular Dynamics). Bands corresponding to phosphatidic acid were identified by co-migration with a lipid standard, and were scraped from the TLC plates and radioactivity quantified by liquid scintillation counting (PerkinElmer Life Sciences).
Assay for triacylglycerol lipase activity

To study the effect of recombinant CGI-58 on triacylglycerol lipase activity of ATGL, 50 µg Sf9 cell extract containing recombinant ATGL was mixed with up to 1 µg of purified recombinant CGI-58 in a final volume of 100 µL 0.1 M potassium phosphate, pH 7.0, before mixing with 100 µL substrate containing 330 µM [9,10-^3^H]triolein emulsified with 145 µM phosphatidylcholine:phosphatidylinositol (3:1) (23). Reactions were incubated at 37°C for 1 hour and terminated by the addition of 3.25 mL methanol:chloroform:heptane [10:9:7 (v:v:v)] and 1.05 mL 0.1 M potassium carbonate, 0.1 M boric acid, pH 10.5. Reactions were centrifuged at 800 x g for 20 minutes at room temperature, after which 1 mL of each top phase was removed and radioactivity quantified by liquid scintillation counting.

Protein-lipid overlay assays

PIP Strips (P-6001), PIP Arrays (P-6100), Membrane Lipid Strips (P-6002), or Sphingo Strips (P-6000) (Echelon, Inc.) were incubated in 5% nonfat dry milk in PBS with 0.1% Tween-20 for 1 hour at room temperature, followed by 12.8 nM (0.5 µg/ml) partially purified recombinant 12-His-tagged CGI-58 in 5% milk in PBS for 1 hour at room temperature. Membranes were rinsed 3 times in PBS with 0.1% Tween-20, followed by incubation with either rabbit polyclonal anti-CGI-58 antiserum or mouse anti-6-His antibody for 1 hour at room temperature. Membranes were again rinsed 3 times followed by incubation with peroxidase-conjugated anti-rabbit or anti-mouse IgG for 1 hour and further rinses. Membranes were developed using enhanced chemiluminescence reagent (Pierce) and then exposed to X-ray film.

Immunoblotting

Immunoblotting of recombinant proteins was performed as described previously (11).
Statistical Analysis

Data representing the means of duplicate or triplicate samples ± standard deviation were analyzed using GraphPad Prism 6 software. Variance was measured using either one-way or two-way ANOVA, followed by Tukey’s multiple comparisons post-hoc test, or Bonferroni’s multiple comparisons post-hoc test, respectively.
Results

Prediction of catalytic residues for LPAAT activity of CGI-58

We and others had previously reported that recombinant CGI-58 has LPAAT activity in vitro (10,11). We embarked on a project to identify the active site residues responsible for enzyme activity. All previously characterized LPAATs contain the conserved proposed active site motif of HX₄D (24). The mouse CGI-58 cDNA sequence includes this motif as HYVYAD with a histidine residue in position 329 (H329) and aspartate in position 334 (D334); hence, we and others have hypothesized that these amino acids comprise active site residues (10,11). These residues are conserved in CGI-58 sequences from multiple species, including C. elegans. Since the crystal structure of CGI-58 has not yet been solved, we developed a hypothetical model of CGI-58 structure to gain a better understanding of the spatial positioning of these proposed active site residues.

Development of a hypothetical model of CGI-58 structure

To generate a hypothetical model of CGI-58 structure, the predicted primary amino acid sequence for mouse CGI-58 was submitted to the publically accessible program PHYRE (15), as well as several other modeling programs. Models that were generated were then subjected to analysis by SAVES (18,17,16,19,20) to determine stability of the predicted structures. The most highly ranked model (Figure 1) was calculated using the primary amino acid sequence of CGI-58 threaded through the crystal structure of a putative hydrolase (2632844) from Bacillus subtilis (Protein Data Bank ID: 2R11).

Using this model, the positions of H329 and D334 were assessed (Figure 1); H329 is located within a hydrophobic pocket in the protein interior, whereas D334 is located on the protein surface, approximately 12.5 angstroms from H329. In previously described acyltransferases, active site residues are located within 2.5 to 4 angstroms of each other to mediate transfer of electrons between catalytic amino acids and substrate (25). Thus, based on the theoretical...
model, it appears unlikely that H329 and D334 are positioned optimally to serve as catalytic residues. Alternatively, the amino acid sequence of CGI-58 contains 20 acidic residues and 4 histidine residues that are conserved among multiple species, and could serve as catalytic amino acids. Since CGI-58 is a member of the lipase subfamily of \( \alpha/\beta \) hydrolase fold domain proteins we reasoned that the conserved GXSXG sequence (GXNXG in CGI-58), which typically harbors a catalytic serine residue, should be found in an active site cleft or pocket, with catalytic acidic and basic residues positioned near N155. In the model (Figure 1), N155 is located within a pocket near two histidine residues, H154 and H329, and one glutamate residue, E179. No other obvious acid-base pairs residing in potential catalytic pockets were predicted by the structural model of CGI-58. Each of these residues was selected for mutagenesis studies to test whether it serves a function in catalysis of LPAAT activity.

**Mutation of predicted catalytic residues failed to reduce LPAAT activity**

Site-directed mutagenesis was used to substitute alanine residues for H329, H154, D334, and E179 in CGI-58 using the previously assembled 12-His CGI-58 cDNA in the pET28a vector (11). Unmodified (WT) recombinant 12-His CGI-58 and the various mutated variants were expressed in BL21(DE3) *E. coli*, partially purified using cobalt affinity chromatography, and tested in vitro for LPAAT activity. Each partially purified protein showed dose-dependent increases in LPAAT activity (data not shown). By Coomassie staining of SDS-PAGE gels (Figure 2A), recombinant 12-His CGI-58 was the major protein in each preparation, but varying levels of additional contaminant proteins were observed. These studies suggested that either we had failed to identify the catalytic residues of CGI-58 or LPAAT activity was due to a contaminant protein(s) from bacterial lysates.

To determine whether bacterial proteins with LPAAT activity can bind to and elute from metal affinity resins for 6-His fusion proteins, bacterial lysates prepared from BL21(DE3) cells harboring empty pET-28a vector were incubated with either nickel or cobalt affinity resins.
Proteins eluted from the affinity resins showed dose-dependent increases in LPAAT activity in the absence of CGI-58 (not shown). Moreover, in the absence of a 6-His fusion protein, eluted proteins showed a prominent band at the position of approximately 27 kDa on Coomassie-stained SDS-PAGE gels, as well as several other less abundant proteins (Figure 2B). We suspected that the 27 kDa band contained plsC, the sole bacterial LPAAT (26). We next pursued a strategy to assess CGI-58 LPAAT activity in the absence of plsC contamination.

Assessment of CGI-58 activity in the absence of plsC

To investigate the contribution of plsC to LPAAT activity of partially purified 12-His-CGI-58, we acquired the *E. coli* strain SM2-1 that lacks plsC activity (27), and used a lysogenization procedure to create the SM2-1(DE3) strain for IPTG-induced expression of T7 RNA polymerase to drive expression of 12-His-CGI-58 in the pET-28a vector. As a positive control, 6-His tagged plsC (in pET-28a) was expressed in SM2-1(DE3) cells. SM2-1 cells are growth restricted at 42°C, but grow well at 28°C. In previous studies of SM2-1 cells, LPAAT activity was undetectable in membrane fractions of cells grown at permissive temperatures, or following a shift to restrictive temperatures (27). Moreover, lysophosphatidic acid accumulates in SM2-1 cells incubated under both permissive and restrictive growth conditions (27), supporting the observation of defective LPAAT activity in the absence of plsC activity. SM2-1(DE3) cells for experiments were grown at 37°C prior to lysis of cells. Lysates of SM2-1(DE3) cells expressing 12-His-CGI58, 6-His-plsC, or empty pET-28a vector were tested in vitro for LPAAT activity. The only lysates that promoted the production of phosphatidic acid were from cells expressing 6-His-plsC; lysates with recombinant CGI-58 lacked LPAAT activity (Figure 3). Lysates from SM2-1(DE3) cells expressing either the empty pET-28a vector or 12-His-CGI-58 lacked LPAAT activity. These results suggest that LPAAT activity previously attributed to CGI-58 is due to the binding and elution of bacterial plsC from metal affinity resins.
Assessment of GST-CGI58 activity

To pursue an alternate strategy to test putative LPAAT activity of recombinant CGI-58, CGI-58 was expressed in BL21(DE3) E. coli as fusion protein with GST appended to the amino terminus (GST-CGI-58). When GST-CGI-58 was purified using a glutathione sepharose column, the eluted protein was unstable; low molecular weight degradation products were observed in Coomassie-stained SDS-PAGE gels. To test LPAAT activity of GST-CGI-58 without interference from endogenous plsC, cell lysates were centrifuged at 16,000 x g to remove bacterial membranes containing plsC. GST-CGI-58 remained soluble in the supernatant (Figure 4A), but displayed no LPAAT activity (Figure 4B). In contrast, whole cell lysates of BL21(DE3) E. coli promoted the formation of phosphatidic acid (Figure 4B), presumably due to LPAAT activity of endogenous plsC.

Assessment of 12-His-CGI-58 without plsC contamination

We modified our purification protocol to reduce co-elution of bacterial proteins over metal affinity resins. After centrifugation to remove the majority of membranes, cell lysates were loaded onto the cobalt affinity resin in solution containing 20 mM imidazole; the column was then washed with a solution containing a higher concentration (100 mM) of imidazole prior to elution with 250 mM imidazole. These conditions improved the purity of eluted 12-His-CGI-58, notably reducing the contaminant band at 27 kDa (Figure 5A). Preparations of 12-His-CGI-58 isolated from either SM2-1(DE3) or BL21(DE3) using this protocol lacked LPAAT activity; in contrast, proteins eluted under previous conditions (20 mM imidazole wash) promoted the formation of phosphatidic acid (Figure 5B), likely due to contamination of the preparations with plsC. These preparations of 12-His CGI-58 were further tested for the ability to co-activate recombinant ATGL-mediated TAG hydrolysis; all preparations showed equivalent increases in the release of radioactive fatty acids from phospholipid emulsions of radiolabeled TAG in the presence of recombinant ATGL (Figure 5C), indicating that recombinant CGI-58 purified by
either protocol is competent to activate ATGL-mediated lipolysis.

**Human CGI-58 expressed in yeast fails to increase LPAAT activity of cell-free extracts**

In a previous study, human CGI-58 (hCGI-58) was overexpressed in yeast and promoted increased $^{32}$P-orthophosphate incorporation into phospholipids including phosphatidylcholine and phosphatidylethanolamine (10). In that study, cell-free extracts of yeast expressing hCGI-58 showed modestly increased LPAAT activity relative to extracts lacking CGI-58. To re-examine whether hCGI-58 expressed in yeast has LPAAT activity, we expressed hCGI-58 in a wild-type strain of yeast and also a deletion strain lacking the endogenous yeast LPAAT, *ict1Δ*. Cell-free extracts from both strains of yeast showed comparable LPAAT activity, whether or not hCGI-58 was expressed (Fig. 6A), supporting the idea that CGI-58 lacks LPAAT activity. Immunoblotting of these cell extracts confirmed the presence of hCGI-58 (Fig. 6B).

**Recombinant CGI-58 binds PIPs**

We had previously reported that recombinant CGI-58 binds lipids, including lysophosphatidic acid and various species of fatty acyl-CoA, substrates for the LPAAT reaction (11). We revisited the binding of lipids to CGI-58 using lipid overlay assays with more highly purified preparations of recombinant CGI-58. Membranes containing 100 pmol per spot of a wide variety of lipid species, including phospholipids, lysophospholipids, phosphorylated phosphatidylinositol, TAG, diacylglycerol, cholesterol, and sphingolipids, were incubated with recombinant CGI-58 prior to immunoblotting for CGI-58 or the 12-His tag. Strong signals for CGI-58 binding were detected for PI(3)P and PI(5)P, with somewhat weaker signals detected for PI(4)P and PI(3,5)P$_2$ (Figure 7A). No CGI-58 binding was detected for lysophosphatidic acid, or other polar or neutral lipids. The relative affinity of lipid binding was assessed by incubating recombinant CGI-58 with a PIP array containing 1.56 to 100 pmol of various PIPs. CGI-58 bound to PI(3)P and PI(5)P in a dose-dependent manner with higher affinity than for PI(4)P and PI(3,5)P$_2$ (Figure 7B). To
confirm that observed signals were due to binding of CGI-58 to lipids on membranes, spots corresponding to recombinant CGI-58 bound to PI(3)P and PI(5)P were cut from membranes and proteins were eluted by boiling the membranes in 2X Laemmli’s sample buffer before elution on SDS-PAGE gels and immunoblotting for CGI-58. This assay confirmed binding of CGI-58 to PI(3)P and PI(5)P on membranes (data not shown).

We next investigated potential consequences of PIP binding on CGI-58-mediated co-activation of ATGL. Recombinant CGI-58 was incubated with 100-fold molar excess of water soluble PIPs with 8-carbon fatty acyl chains; lipids tested included PI(3)P, PI(5)P, and PI(4,5)P_2, the latter serving as a PIP that is not bound by CGI-58. The mixtures were then added to recombinant ATGL and TAGs emulsified with phospholipids for assessment of TAG hydrolysis. Recombinant CGI-58 increased TAG hydrolysis approximately 3.5-fold, whether or not PIPs were present (Figure 7C). These data suggest that PIP binding does not impact CGI-58 interaction with ATGL.
Discussion

The major finding of this study is that recombinant CGI-58 lacks LPAAT activity. Previously reported LPAAT activity (10,11) is most likely due to co-elution of plsC, the sole LPAAT in E. coli (26), over the metal affinity resin used to purify 12-His CGI-58. Several new lines of evidence provide support for the conclusion that CGI-58 is not an LPAAT: 1) in vitro LPAAT assays of cell extracts from SM2-1(DE3) E. coli expressing recombinant CGI-58, but lacking endogenous plsC, show absence of LPAAT activity, 2) bacterial cell extracts with recombinant GST-CGI-58 lack LPAAT activity when membranes with endogenous plsC are removed by centrifugation, 3) purification of recombinant 12-His-CGI-58 over metal affinity resins yields CGI-58 free of LPAAT activity when E. coli membranes are removed by centrifugation and stringent wash conditions are employed prior to elution of protein, and 4) cell-free extracts from yeast fail to show increased LPAAT activity when hCGI-58 is expressed. Consistent with these findings, a theoretical model of CGI-58 structure shows that the histidine and aspartate residues in the putative LPAAT active site motif of HXXXXD are not in close proximity and hence, are unlikely to contribute to catalytic activity.

This is not the first time that researchers have attributed LPAAT activity of endogenous plsC to recombinant proteins purified from bacterial lysates. In the early 2000s, endophilins A1 and B1 (28,29) and CtBP/BARS (carboxyl-terminal binding protein/brefeldin A-ribosylated substrate (30) were proposed to have LPAAT activity based on in vitro assays of recombinant proteins purified from E. coli. Endophilins and CtBP/BARS have been implicated in either the formation of membrane vesicles or in membrane fission (31,32,33,34). The identification of LPAAT activity in these proteins raised the attractive hypothesis that LPAAT activity to reduce levels of lysophosphatidic acid (an inverted cone-shaped lipid) and increase formation of phosphatidic acid (a cone-shaped lipid) contributed to the mechanisms by which vesicular membranes were remodeled by altering membrane curvature (35,29,36,30). However, a later study showed that the previously observed LPAAT activity was due to the co-purification of
bacterial plsC with the recombinant proteins (37). In these studies, plsC had likely been a contaminant in preparations of recombinant proteins purified using both metal affinity resins and glutathione affinity resins.

Co-purification of plsC with recombinant fusion proteins may be due to physical characteristics of the bacterial LPAAT. Firstly, plsC is a membrane-associated protein in bacteria (26), so the presence of membrane fragments in cell lysates will increase the likelihood of co-purification of plsC with the protein of interest. We have found that centrifugation of bacterial lysates for 20 minutes at 21,000 x g is sufficient to remove most bacterial membranes containing plsC, leaving soluble recombinant fusion proteins in the supernatant, and reducing subsequent plsC contamination of proteins eluted from column chromatography steps. Secondly, plsC is a 27-kDa protein of 245 amino acids with a calculated isoelectric point of 9.61 and estimated charge of 12.0 at pH 7.5; the amino acid composition of plsC may increase affinity of the protein for metal affinity resins as well as increase retention of the contaminant on cation exchange resins. In our hands, increased stringency of wash conditions was required to reduce co-purification of plsC with recombinant fusion proteins. Finally, it is possible that plsC may have affinity for some recombinant proteins, leading to co-purification of the contaminant with the protein of interest.

Several prior studies of CGI-58 in mice have provided intriguing data showing that knockdown of CGI-58 alters the phospholipid content of liver. When CGI-58 anti-sense oligonucleotides were administered to mice, near complete knockdown of CGI-58 in liver was observed, accompanied by reductions in hepatic phosphatidic acid, phosphatidylcholine, and phosphatidylethanolamine, with major increases in phosphatidylglycerol and sphingomyelin content (12,13). Additional experiments showed a modest reduction in overall LPAAT activity in liver when CGI-58 ASO-treated animals were injected with tumor necrosis factor α (13). Our data suggest that these observed effects were not due to reduction of LPAAT activity, but instead, changes in phospholipid levels may be due to reduced ATGL-mediated hydrolysis of triacylglycerols leading to reduced provision of substrates for phospholipid synthesis (11).
Additionally, since CGI-58 ASO treatment significantly altered cytokine production (13), reduction of hepatic LPAAT activity in response to injection of an inflammatory mediator may have been downstream of these signals. Alternatively, CGI-58 may serve additional as yet undiscovered functions in lipid metabolism.

We had previously reported that CGI-58 bound lysophosphatidic acid and several species of fatty acyl-CoA (11), substrates for the LPAAT reaction. After modifying our purification protocol to reduce levels of contaminant proteins, we re-visited lipid binding of recombinant CGI-58 using protein-lipid overlay assays. In these assays, recombinant CGI-58 failed to bind lysophosphatidic acid and several other neutral and polar species of lipids, but surprisingly, displayed dose-dependent binding to PI(3)P and PI(5)P with estimated apparent dissociation constants of 6 µM. The apparent affinity of CGI-58 for these lipids is similar to previously published dissociation constants of several PIP-binding proteins (38,39,40,41). The functional significance of PIP binding is unknown for CGI-58. Recombinant CGI-58 co-activated the TAG hydrolytic activity of ATGL equally well in the presence or absence of 100-fold molar excess concentrations of water soluble species of PI(3)P and PI(5)P, so PIP binding neither increases nor decreases this co-activation function.

PIPs facilitate protein binding to membranes for a wide variety of components of signaling pathways and subcellular trafficking mechanisms for proteins, including sorting nexins, protein kinase B (Akt), oxysterol binding proteins, and phospholipase D1 (42,43,44). Several protein domains interact with PIPs including pleckstrin homology (PH) domains, phox homology (PX) domains and FYVE domains. The amino acid sequence of CGI-58 does not obviously include any of these domains, although there is some variability in the amino acid sequences that fold into these tertiary structures, making them difficult to identify unambiguously. It is possible that PIP binding influences subcellular localization of CGI-58. In cultured adipocytes, CGI-58 localizes to lipid droplets in a perilipin 1-dependent manner under basal (corresponding to fed) conditions (14,45), but disperses into the cytoplasm when lipolysis is stimulated following
activation of β-adrenergic signaling (14). In other types of cultured cells, CGI-58 localizes to both the cytoplasm and surfaces of lipid droplets; mechanisms that control the movement of CGI-58 between these compartments have not been identified, although it has been proposed that CGI-58 assists in the recruitment of ATGL to lipid droplets (46). The presence of PIPs may assist in recruitment of CGI-58 to lipid droplets; however, although PI has been identified as a component of lipid droplets (47,48), to date, there has been no specific assessment of the PIP content of lipid droplets. Interestingly, PI3-kinase, an enzyme that converts PI to PI(3)P, has been identified on the lipid droplets of human leukocytes (49), suggesting that PI(3)P may be formed on lipid droplets, at least in some types of cells. The functional importance of CGI-58 binding of PI(3)P and PI(5)P requires further study.

In summary, previously identified LPAAT activity of recombinant CGI-58 is due to the contamination of partially purified protein with the bacterial acyltransferase, plsC. Alteration of purification methods to reduce plsC contamination eliminates LPAAT activity, without affecting the capacity of recombinant CGI-58 to co-activate TAG hydrolysis catalyzed by ATGL. Finally, recombinant CGI-58 fails to bind lysophosphatidic acid immobilized on membranes, while binding PI(3)P and PI(5)P with high affinity.
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Abbreviations: ATGL, adipose triglyceride lipase; CGI-58, Comparative Gene Identification 58; CDS, Chanarin-Dorfman Syndrome; GST, glutathione-S-transferase; IPTG, isopropyl-β-D-1-thiogalactopyranoside; LPA, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; PIP, phosphatidylinositol phosphate; PI(3)P, phosphatidylinositol 3-phosphate; PI(5)P, phosphatidylinositol 5-phosphate; TAG, triacylglycerol
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CGI-58 MISLOCALIZATION IN CHANARIN-DORFMAN SYNDROME. J. Biol. Chem. 279: 30490-30497 PM:15136565.


Figure 1. Theoretical model of CGI-58 structure. The amino acid sequence of CGI-58 was threaded through the solved crystal structure of a putative hydrolase (2632844) from Bacillus subtilis (Protein Data Bank ID: 2R11). Hydrophobic residues are depicted in red; hydrophilic residues are depicted in blue. Putative acyltransferase active site residues H329 and D334 are depicted in cyan and magenta, respectively. H329 is located in an internal pocket, whereas D334 is located 12.5 angstroms away on the exterior surface of the protein. N155 within the conserved motif GXNXG is depicted in green, while H154, H329 and E179 surround N155 and are depicted in yellow, cyan, and white respectively.

Figure 2. Preparations of recombinant 12-His-CGI-58 show contaminants after metal affinity resin chromatography. A) Coomassie stained SDS-PAGE gels of recombinant 12-His-CGI-58 and mutated variants eluted from cobalt affinity resin. Lanes depict molecular weight markers (MW; lane 1), 1 µg 12-His CGI-58 (lane 2), H329A substituted 12-His-CGI-58 (lane 3), and D334A substituted 12-His-CGI-58 (lane 4). B) Coomassie stained SDS-PAGE gel of BL21(DE3) lysate lacking 12-His-CGI-58 (lane 1) and recombinant 12-His-CGI-58 (lane 2) following elution over cobalt affinity resin. Arrowheads indicate position of stained CGI-58.

Figure 3. Recombinant 12-His-CGI-58 in SM2-1(DE3) bacterial cell lysate lacks LPAAT activity. A) Immunoblot of SM2-1(DE3) bacterial cell lysates probed with anti-6-His antibody. Lanes depict 20 µg lysate with 6-His-plsC (lane 1), empty pET-28a vector (lane 2), 12-His-CGI-58 (lane 3), and 100 ng partially purified 12-His-CGI-58 (lane 4). 6-His-plsC elutes at approximately 29-kDa in lane 1; the 29-kDa band in lane 3 is likely a degradation product of 12-His-CGI-58. B) Phosphorimagher scan of a TLC plate with eluted lipid extracts from LPAAT activity assay. Whole cell lysates of SM2-1(DE3) cells transformed with empty pET-28a vector (lanes 3-8), or expressing 12-His-CGI-58 (lanes 9-14) or 6-His-plsC (lanes 15-20) were assayed.
for LPAAT activity using $[^14]C$oleoyl-CoA and lysophosphatidic acid in duplicate reactions with 5, 10, or 20 µg of cell protein. Radiolabeled phosphatidic acid detected in lanes 15-20 corresponded to unlabeled phosphatidic acid standards eluted on the same TLC plate (PA; arrowhead). Only lanes with 6-His-plsC showed the production of radiolabeled phosphatidic acid. Lanes 1 and 2 show lipid extracts of reaction mixtures lacking protein.

**Figure 4. GST-CGI-58 lacks LPAAT activity when bacterial membranes are removed by centrifugation.** A) Immunoblot of GST-CGI-58 in BL21(DE3) lysates before (lane 1) and after (lane 2) centrifugation at 16,000 $x$ g to remove cell membranes. Blot was probed with anti-GST antibodies. GST-CGI-58 remains in the supernatant following centrifugation. B) Phosphorimager scan of a TLC plate with eluted lipid extracts from an LPAAT activity assay. Whole cell lysates of BL21(DE3) cells expressing GST-CGI-58 (lanes 3-8) and supernatant following centrifugation of lysates (lanes 9-14) were assayed for LPAAT activity in duplicate reactions with 2.5, 5, or 10 µg protein. Only lanes with whole cell lysates showed production of radiolabeled phosphatidic acid; centrifugation removed LPAAT activity. Lanes 1 and 2 show lipid extracts of reaction mixtures lacking protein.

**Figure 5. Improved purification of 12-His-CGI-58 reduces levels of contaminant proteins and LPAAT activity with little effect on its ATGL co-activator function.** A) Coomassie stained SDS-PAGE gel of molecular weight markers (lane 1), 1 µg of 12-His-CGI-58 purified from SM2-1(DE3) cells (lane 2), 12-His-CGI-58 purified from BL21(DE3) cells with 100 mM imidazole wash step (lane 3), and 12-His-CGI-58 purified from BL21(DE3) cells with 20 mM imidazole wash step (lane 4; BL21(DE3)*). Arrowhead indicates CGI-58. B) Phosphorimager scan of TLC plate with eluted lipid extracts from LPAAT activity assay. 12-His-CGI-58 purified from SM2-1(DE3) cells (lanes 3-8), BL21(DE3) cells using 100 mM imidazole wash step (lanes 9-14), and BL21(DE3) cells using the 20 mM imidazole wash step (lanes 15-20) were assayed...
for LPAAT activity in duplicate reactions with 0.25, 0.5, 1 µg protein. Only lanes from BL21(DE3) purifications with the 20 mM imidazole wash step showed production of radiolabeled phosphatidic acid. Lanes 1 and 2 show lipid extracts of reaction mixtures lacking protein. Arrowhead shows elution position for phosphatidic acid (PA). C) Each of the 3 preparations of 12-His-CGI-58 co-activated TAG hydrolytic activity of ATGL similarly. Post-nuclear Sf9 cell extracts containing recombinant 6-His-ATGL (50 µg) were added to 0.25, 0.5, or 1 µg purified 12-His-CGI-58 and [3H]triolein emulsified with phospholipids. Fatty acids were extracted and quantified. (●) CGI-58 purified from SM2-1(DE3) cells; (▲) CGI-58 purified from BL21(DE3) cells using a 20 mM imidazole wash step; (■) CGI-58 purified from BL21(DE3) cells using a 100 mM imidazole wash step. Data are the means ± standard deviation of duplicate reactions and depict one representative experiment out of six. Data were analyzed by two-way ANOVA; preparations of CGI-58 were not significantly different from each other, but all samples containing CGI-58 were significantly different from samples containing only ATGL (p < 0.001).

Figure 6. Cell-free extracts of yeast expressing human CGI-58 show comparable LPAAT activity to extracts lacking CGI-58. A) Cell-free extracts (100,000 x g supernatants) of a wild-type strain (WT; BY4741) or a deletion strain lacking ICT1 (ict1Δ) with and without hCGI-58 were tested for LPAAT activity. The expression of hCGI-58 does not increase LPAAT activity. Data are the means ± standard error of the means of average values from 3 experiments, each with duplicate samples. B) Immunoblot showing hCGI-58 expression in 100,000 x g supernatants from WT and ict1Δ cells expressing hCGI-58 (lanes 3 and 4, respectively), but not in control cell extracts (lanes 1 and 2). Lanes show band for the Protein A-hCGI-58 fusion protein (arrowhead; upper band) and cleavage product consistent with the size of hCGI-58 (lower band). Immunoblots were probed with anti-human ABHD5 antibody.
Figure 7. 12-His-CGI-58 binds PI(3)P and PI(5)P with weaker binding to PI(4)P and PI(3,5)P$_2$. A) 12-His-CGI-58 (12.8 nM) was incubated with membranes containing 100 pmol/spot of various lipids including lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingosine-1-phosphate (S1P), phosphatidic acid (PA), phosphatidyserine (PS), and various phosphoinositides (eg. PI(3)P). CGI-58 was detected using anti-CGI-58 antiserum. B) 12-His-CGI-58 (12.8 nM) was incubated with membranes containing 1.56 to 100 pmol/spot of various lipids, followed by detection with anti-CGI-58 antiserum. C) Preincubation of 12-His-CGI-58 with 100-fold molar excess PI(3)P, PI(5)P or PI(4,5)P$_2$ with 8-carbon acyl chains does not alter CGI-58 function as a co-activator of ATGL. Post-nuclear Sf9 cell extracts containing recombinant 6-His-ATGL (50 µg) were added to 1 µg purified 12-His-CGI-58 (with or without PIPs) and [$^3$H]triolein emulsified with phospholipids. Fatty acids were extracted and quantified. Data are the means ± standard deviation of duplicate reactions and depict one representative experiment out of three. Data were analyzed by one-way ANOVA; data with the same superscript (a or b) were not different from each other, but all samples containing ATGL and CGI-58 were significantly different from samples containing only ATGL (p < 0.001). Where error bars are not visible, they are contained within the symbol.
Figure 3

### Table

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**Figure A:**
- Lysates
- kDa markers: 43, 26, 17
- CGI-58
- plsC

**Figure B:**
- PA

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Figure 4