Comparative gene identification 58/α/β hydrolase domain 5 lacks lysophosphatidic acid acyltransferase activity

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Abstract Mutations in the gene encoding comparative gene identification 58 (CGI-58)/α/β hydrolase domain 5 (ABHD5) cause Chanarin-Dorfman syndrome, characterized by excessive triacylglycerol storage in cells and tissues. CGI-58 has been identified as a coactivator of adipose TG 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 WT 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 Escherichia 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 glutathione-S-transferase 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]. Prebinding CGI-58 with PI(3)P or PI(5)P did not alter its coactivation of ATGL in vitro. In summary, purified recombinant CGI-58 that is functional as an ATGL coactivator lacks LPAAT activity.—McMahon, D., A. Dinh, D. Kurz, D. Shah, G-S. Han, G. M. Carman, and D. L. Brasaemle. Comparative gene identification 58/α/β hydrolase domain 5 lacks lysophosphatidic acid acyltransferase activity. J. Lipid Res. 2014. 55: 1750–1761.

Supplementary key words phosphatidylinositol 3-phosphate • phosphatidylinositol 5-phosphate • adipose triglyceride lipase • Chanarin-Dorfman syndrome • neutral lipid storage disorder

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 (2–7). 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 (GXSGXG) that normally harbors the nucleophilic component of the catalytic triad (1). Identification of CGI-58 as a coactivator of the widely expressed adipose TG 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 coactivation of ATGL.

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In 2008, Ghosh and coworkers (10) reported lysophos- 
phatic acid acyltransferase (LPAAT) activity of recombi-
nant CGI-58 in assays conducted in vitro; we later con-
firmed this activity for recombinant CGI-58 (11). Because phospha-
tidic acid (PA), 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 in 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 be-
tween CGI-58 of various species, to predict potential cata-
lytic 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 *Escherichia 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 copurification of plsC, the sole bacterial LPAAT, over 
cobalt affinity resin. Lipid binding activity of CGI-58 was 
revisited using highly purified recombinant protein; previ-
ously reported binding of lysophosphatic acid (LPA) 
was not observed, but novel binding activity toward phosphati-
dylinositol 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 previ-
ously reported (14). The cDNA for mouse ATGL (IMAGE: 
5150311) was submitted to the online Protein Homology/analogy 
Recognition Engine (PHRE) version 0.2 (15). Resulting models 
were then evaluated using the Structural Analysis and Verifica-
tion Server (SAVES) (http://nihserver.mbi.ucla.edu/SAVES). The 
SAVES program includes five programs, including PROCHECK 
(16), WHAT_CHECK (17), ERRAT (18), VERIFY3D (19), and 
PROVE (20), which assess the structural stability of the comput-
gerated models. The highest-ranked model was based on a pu-
tative hydrolase (2629344) 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 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 (PHRE) version 0.2 (15). Resulting models 
were then evaluated using the Structural Analysis and Verifica-
tion Server (SAVES) (http://nihserver.mbi.ucla.edu/SAVES). The 
SAVES program includes five programs, including PROCHECK 
(16), WHAT_CHECK (17), ERRAT (18), VERIFY3D (19), and 
PROVE (20), which assess the structural stability of the comput-
gerated models. The highest-ranked model was based on a pu-
tative hydrolase (2629344) 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 ADE3 lysogenization 
kit to integrate the ADE3 prophage into the SM2-1 *E. coli* chromo-
some 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 3 or 3 h, respectively. Cells were centrifuged at 4,000 
g at 10 min for 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 DTT, and prote-
ase inhibitors (Roche Complete, EDTA free), followed by 
icubation on ice for 30 min. Cells were disrupted with glass 
beads in a Bead-Beater® (Biospec Products Inc.) chamber sur-
rounded by ice water; cells were disrupted with 10 cycles of 
15 s blending with 2 min intermittent cooling. lysed cells were 
centrifuged at 21,000 g for 20 min at 4°C. Clarified superna-
tant was incubated with 0.5 ml TALON His-Tag Purification 
Resin for 1.5 h at 4°C prior to brief centrifugation at 800 g 
at 4°C. The resin pellet was resuspended in lysis buffer with

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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. B121(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 h. Cells were centrifuged at 4,000 g for 10 min at 4°C, the supernatant was removed, and pelleted cells were lysed in PBS with 0.5 mg/ml lysozyme and protease inhibitors. A fraction of lysate was centrifuged at 16,000 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′-GCCACATGTGTCGGAGGG-3′) and reverse (5′-TTATGGT-GATGTGATGTGATGTAGTCGGACAGCGGAGGAG-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-6His 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 minipreps, 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 Miniprep 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 h after transfection and centrifuged at 800 g for 10 min 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 h 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 g for 10 min 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 min on ice. The resuspended cells were disrupted by passing them through a 27.5 gauge syringe 10 times. Lysates were centrifuged at 800 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′-GCCACATGTGTCGGAGGG-3′) and reverse (5′-CCGTCGAGTCTGACAGTGTC-3′) primers and was subcloned into the NdeI and Xhol restriction sites of the pYES2 vector (Invitrogen) containing the Protein A sequence derived from Psa-DGK1 (21). The internal NdeI restriction site of the human CGI-58 (hCGI-58) cDNA was removed by introduction of a silent mutation using the QuikChange® Site-Directed Mutagenesis kit (Strategene).

Yeast strains BY4741 (22) and ietΔ (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ietΔ:kanMX) containing pYES2-Tra-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 h 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 s with intermittent cooling. Cell lysates were centrifuged for 10 min at 1,500 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 h at 100,000 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 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 with addition of 0.1N HCl in methanol-chloroform-MgCl2 [1:2.5:3 (v/v/v)] and centrifugation at 800 g for 10 min 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 PA were identified by comigration with a lipid standard and were scraped from the TLC plates, and radioactivity was quantified by liquid scintillation counting (PerkinElmer Life Sciences).

Assay for TAG lipase activity

To study the effect of recombinant CGI-58 on TAG 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 [9,10-2H] triolein emulsified with 145 µM PC-PI (3:1) (23). Reactions were incubated at 37°C for 1 h 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 g for 20 min 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 h 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 h at room temperature. Membranes were rinsed three 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 h at room temperature. Membranes were again rinsed three times followed by incubation with peroxidase-conjugated anti-rabbit or anti-mouse IgG for 1 h 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 HX,D (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 Caenorhabditis elegans. Because 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 (16–20) to determine stability of the predicted structures. The most highly ranked model (Fig. 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 (Fig. 1); H329 is located within a hydrophobic pocket in the protein interior, whereas D334 is located on the protein surface, ~12.5 Å from H329. In previously described acyltransferases, active site residues are located within 2.5 to 4 Å 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. Because CGI-58 is a member of the lipase subfamily of α/β 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 (Fig. 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...

Fig. 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 Å 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.
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 (Fig. 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 ~27 kDa on Coomassie-stained SDS-PAGE gels, as well as several other less abundant proteins (Fig. 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 in 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, LPA 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 expressing 12-His-CGI-58, 6-His-plsC, or empty pET-28a vector were tested in vitro for LPAAT activity. The only lysates that promoted the production of PA were from cells expressing 6-His-plsC; lysates with recombinant CGI-58 lacked LPAAT activity (Fig. 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-CGI-58 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 N 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 × g to remove bacterial membranes containing plsC. GST-CGI-58 remained soluble in the supernatant (Fig. 4A) but displayed no LPAAT activity (Fig. 4B). In contrast, whole cell lysates of BL21 (DE3) E. coli promoted the formation of PA (Fig. 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 coelution 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 (Fig. 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 PA (Fig. 5B), likely due to contamination of the preparations with plsC. These preparations of 12-His-CGI-58 were further tested for the ability to coactivate 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 (Fig. 5C), indicating that recombinant CGI-58 purified by either protocol is competent to activate ATGL-mediated lipolysis.

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

In a previous study, hCGI-58 was overexpressed in yeast and promoted increased $^{32}$P-orthophosphate incorporation into phospholipids including PC and phosphatidyl-ethanolamine (PE) (10). In that study, cell-free extracts of yeast expressing hCGI-58 showed modestly increased LPAAT activity relative to extracts lacking CGI-58. To reexamine whether hCGI-58 expressed in yeast has LPAAT activity, we expressed hCGI-58 in a WT strain of yeast and also a deletion strain lacking the endogenous yeast LPAAT, i.e., $\Delta$. 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 LPA 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 PI, 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$_2$ (Fig. 7A). No CGI-58 binding was detected for LPA or other polar or neutral lipids. The relative affinity of lipid binding was assessed by incubating recombinant CGI-58 with a PIP array containing [14C]oleoyl-CoA and LPA in duplicate reactions with 5, 10, or 20 μg of cell protein. Radiolabeled PA detected in lanes 15–20 corresponded to unlabeled PA standards eluted on the same TLC plate. Only lanes with 6-His-plsC showed the production of radiolabeled PA. Lanes 1 and 2 show lipid extracts of reaction mixtures lacking protein.

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Fig. 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 ~29 kDa in lane 1; the 29 kDa band in lane 3 is likely a degradation product of 12-His-CGI-58. B: Phosphorimager 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 [14C]oleoyl-CoA and LPA in duplicate reactions with 5, 10, or 20 μg of cell protein. Radiolabeled PA detected in lanes 15–20 corresponded to unlabeled PA standards eluted on the same TLC plate. Only lanes with 6-His-plsC showed the production of radiolabeled PA. Lanes 1 and 2 show lipid extracts of reaction mixtures lacking protein.
purified from bacterial lysates. In the early 2000s, endophilins A1 and B1 (28, 29) and carboxyl-terminal binding protein/brefeldin A-ribosylated substrate (CtBP/BARS) (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 (31–34). The identification of LPAAT activity in these proteins raised the attractive hypothesis that LPAAT activity to reduce levels of LPA (an inverted cone-shaped lipid) and increase formation of PA (a cone-shaped lipid) contributed to the mechanisms by which vesicular membranes were remodeled by altering membrane curvature (29, 30, 35, 36). However, a later study showed that the previously observed LPAAT activity was due to the copurification of 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. Copurification of plsC with recombinant fusion proteins may be due to physical characteristics of the bacterial LPAAT. First, plsC is a 27 kDa protein of 245 amino acids hydrolysis ∼3.5-fold, whether or not PIPs were present (Fig. 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 coelution 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 used 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 carboxyl-terminal binding protein/brefeldin A-ribosylated substrate (CtBP/BARS) (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 (31–34). The identification of LPAAT activity in these proteins raised the attractive hypothesis that LPAAT activity to reduce levels of LPA (an inverted cone-shaped lipid) and increase formation of PA (a cone-shaped lipid) contributed to the mechanisms by which vesicular membranes were remodeled by altering membrane curvature (29, 30, 35, 36). However, a later study showed that the previously observed LPAAT activity was due to the copurification 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. Copurification of plsC with recombinant fusion proteins may be due to physical characteristics of the bacterial LPAAT. First, plsC is a membrane-associated protein in bacteria (26), so the presence of membrane fragments in cell lysates will increase the likelihood of copurification of plsC with the protein of interest. We have found that centrifugation of bacterial lysates for 20 min at 21,000 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. Second, plsC is a 27 kDa protein of 245 amino acids...
Fig. 5. Improved purification of 12-His-CGI-58 reduces levels of contaminant proteins and LPAAT activity with little effect on its ATGL coactivator 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...
CGI-58 ASO–treated animals were injected with tumor necrosis factor α to reduce provision of substrates for phospholipid synthesis and reduced ATGL-mediated hydrolysis of TAGs leading to reduced overall LPAAT activity in liver when 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.

Several prior studies in 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 (ASOs) were administered to mice, near complete knockdown of CGI-58 in liver was observed, accompanied by reductions in hepatic PA, PC, and PE, 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 TAGs leading to reduced provision of substrates for phospholipid synthesis (11). Additionally, because 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 LPA 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 revisited lipid binding of recombinant CGI-58 using protein-lipid overlay assays. In these assays, recombinant CGI-58 failed to bind LPA 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 (38–41). The functional significance of PIP binding is unknown for CGI-58. Recombinant CGI-58 coactivated 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 coactivation 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, oxysterol binding proteins, and phospholipase D1 (42–44). Several protein domains interact with PIPs including pleckstrin homology domains, phox homology 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, PI 3-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 coactivate TAG hydrolysis catalyzed by ATGL. Finally, recombinant CGI-58 fails to bind LPA immobilized on membranes, while binding PI(3)P and PI(5)P with high affinity.

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**Fig. 7.** 12-His-CGI-58 binds PI(3)P and PI(5)P with weaker binding to PI(4)P and PI(3,5)P₂. A: 12-His-CGI-58 (12.8 nM) was incubated with membranes containing 100 pmol/spot of various lipids including LPA, lysophosphatidylcholine (LPC), PI, PE, PC, sphingosine-1-phosphate (S1P), PA, phosphatidylserine (PS), and various phosphoinositides [e.g., 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)P₂ with 8-carbon acyl chains does not alter CGI-58 function as a coactivator of ATGL. Postnuclear 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 [³H]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.
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