Perilipin A Mediates the Reversible Binding of CGI-58 to Lipid Droplets in 3T3-L1 Adipocytes*

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Perilipins, the major structural proteins coating the surfaces of mature lipid droplets of adipocytes, play an important role in the regulation of triacylglycerol storage and hydrolysis. We have used proteomic analysis to identify CGI-58, a member of the αβ-hydrolase fold family of enzymes, as a component of lipid droplets of 3T3-L1 adipocytes. CGI-58 mRNA is highly expressed in adipose tissue and testes, tissues that also express perilipins, and at lower levels in liver, skin, kidney, and heart. Both endogenous CGI-58 and an ectopic CGI-58-GFP chimera show diffuse cytoplasmic localization in 3T3-L1 preadipocytes, but localize almost exclusively to the surfaces of lipid droplets in differentiated 3T3-L1 adipocytes. The localization of endogenous CGI-58 was investigated in 3T3-L1 cells stably expressing mutated forms of perilipin using microscopy. CGI-58 binds to lipid droplets coated with perilipin A or mutated forms of perilipin with an intact C-terminal sequence from amino acid 382 to 429, but not to lipid droplets coated with perilipin B or mutated perilipin A lacking this sequence. Immunoprecipitation studies confirmed these findings, but also showed co-precipitation of perilipin B and CGI-58. Remarkably, activation of cAMP-dependent protein kinase by the incubation of 3T3-L1 adipocytes with isoproterenol and ibutylmethylxanthine disperses CGI-58 from the surfaces of lipid droplets to a cytoplasmic distribution. This shift in subcellular localization can be reversed by the addition of propanolol to the culture medium. Thus, CGI-58 binds to perilipin A-coated lipid droplets in a manner that is dependent upon the metabolic status of the adipocyte and the activity of cAMP-dependent protein kinase.

Lipid droplets, organelles that store neutral lipids, are found in nearly all types of eukaryotic cells. The most highly studied lipid droplet-associated proteins are members of the PAT family (for perilipin, adipophilin, and TIP47) that coat the lipid droplets of steroidogenic cells (6) that store cholesterol ester precursors for steroid hormone synthesis.

The members of the PAT family comprise the major structural proteins of lipid droplets. Studies in perilipin knockout mice have established an important role for perilipins in regulating lipolysis in adipocytes, and hence, controlling the mass of triacylglycerol deposited in adipose tissue (10, 11). The mechanisms by which perilipins maintain control of adipocyte triacylglycerol metabolism are poorly understood, although recent mutagenesis studies have identified structural motifs important to perilipin function in triacylglycerol storage and lipolysis (12–16). An attractive model consistent with these data suggests that perilipins restrict the access of cytosolic lipases to triacylglycerol stored within lipid droplets, and thus suppress the rate of basal lipolysis under conditions when triacylglycerol storage is favored. When catecholamines activate β-adrenergic receptors, the resultant phosphorylation of perilipin A by cAMP-dependent protein kinase (PKA) on as many as six serines throughout the perilipin sequence facilitates the docking of hormone-sensitive lipase on lipid droplets (17) leading to a dramatically increased rate of lipolysis.

Recent studies (8, 18–21) have expanded the list of lipid droplet-associated proteins to include several with known enzymatic functions in sterol synthesis and lipid metabolism, as well as numerous proteins with unknown functions. Here we report the identification of CGI-58 as a component of lipid droplets isolated from cultured 3T3-L1 adipocytes (41). The name CGI-58 derives from a Comparative Gene Identification initiative that annotated 150 novel transcripts conserved between Caenorhabditis elegans and humans (22). CGI-58 is a member of the PAT family of proteins (18, 19), and includes perilipin, adipophilin, TIP47, and a related but structurally divergent protein, S3-12 (2, 3). Three protein isoforms of perilipins, named perilipins A, B, and C, are translated from alternatively spliced forms of mRNA. Perilipin A and S3-12 localize to lipid droplets in adipocytes (3–5), where they are the most abundant structural proteins of large mature and small nascent lipid droplets, respectively. In contrast, most other types of cells have small lipid droplets covered with adipophilin (6, 7) and TIP47 (1, 8, 9). Perilipins A and C, and adipophilin, coat the lipid droplets of steroidogenic cells (6) that store cholesterol ester precursors for steroid hormone synthesis.

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member of the esterase, thioesterase, and lipase subfamily of \(\alpha/\beta\)-hydrolase fold enzymes (23). The protein sequence includes a highly conserved active site that, in most \(\alpha/\beta\)-hydrolases, includes a nucleophilic serine residue known to be important in catalysis. Interestingly, the CGI-58 sequence encodes an asparagine in place of this catalytic serine (23). Significantly, eight different mutations in CGI-58 were recently identified as causative factors in Chanarin-Dorfman Syndrome (9), a neutral lipid storage disorder (NLSD) characterized by ichthyosis, hepatic steatosis and hepatomegaly, developmental defects, and the accumulation of triacylglycerol-containing lipid droplets in leukocytes, basal keratinocytes, hepatocytes, myocytes, and other cells (23–25). The eight mutations known to lead to NLSD include truncations, null alleles, and three distinct point mutations. This suggests that the lipid storage phenotype of NLSD most likely arises from loss of function of CGI-58, leading to excessive storage of triacylglycerols in lipid droplets.

In this study, we have investigated the tissue distribution of CGI-58 and characterized the localization of both endogenous CGI-58 and a CGI-58-green fluorescent protein (GFP) chimera in 3T3-L1 preadipocytes and differentiated adipocytes. After finding extensive CGI-58 localization to lipid droplets in adipocytes but not preadipocytes, we investigated the interaction of CGI-58 with perilipin by studying 3T3-L1 preadipocytes stably expressing various mutated forms of perilipin A.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fetal bovine serum, fatty acid-free bovine serum albumin, triolein, oleic acid, isobutylmethylkathione (IBMX), insulin, dexamethasone, forskolin, and horseradish peroxidase-conjugated goat anti-\(\alpha\)-rabbit IgG were purchased from Sigma. Geneticin was purchased from Mediatech (Herndon, VA). \(Pfu\) DNA polymerase was purchased from Stratagene. The Expand High Fidelity PCR System was purchased from Roche Applied Science. BCA protein assay reagents were purchased from Pierce. Bodipy 493/503 and Alexa Fluor 546 goat-anti-rabbit IgG were purchased from Molecular Probes (Eugene, OR). Fluorescein- and rhodamine-conjugated goat anti-rabbit or goat anti-guinea pig IgGs were purchased from Jackson Immunoresearch, Inc. (West Grove, PA). Rabbit polyclonal anti-GFP antisera was purchased from Novus Biologicals (Littletown, CO). Mouse tissue mRNA blot arrays were purchased from OriGene Technologies, Inc. (Rockville, MD) and BioChain Institute, Inc. (Hayward, CA).

**Cell Culture**—3T3-L1 preadipocytes and 293T cells were cultured as described previously (26). Confluent monolayers of 3T3-L1 preadipocytes were induced to differentiate into adipocytes by the daily addition of culture medium containing 8 \(\mu\)g/ml insulin, 0.5 mM IBMX, 10 \(\mu\)g/ml insulin, and 10 \(\mu\)M dexamethasone for 72 h followed by daily addition of culture medium containing dexamethasone, but without the other additives for 4–7 more days (6).

**Identification of CGI-58 by Mass Spectrometric Analysis**—The methods used for the isolation of lipid droplets and identification of proteins in lipid droplet preparations by mass spectrometry are described previously (27). Tryptic peptides were analyzed by micro-HPLC-MS/MS analysis using an online MicroPro-HPLC system (Eldex Laboratories) coupled with a LCQ electrospray ionization (ThermoFinnigan) operated in a data-dependent mode for measuring the molecular masses of parent peptides and collecting MS/MS peptide fragmentation spectra. The measured molecular masses of parent peptides and their MS/MS data were used to search the National Center for Biotechnology Information DNA/protein sequence databases using the programs PROBE and BLAST.

**Expression of Recombinant CGI-58 and Production of Polyclonal Antibera in Rabbits**—A murine CGI-58 cDNA was amplified out of 3T3-L1 adipocyte cDNA library (kindly donated by Dr. Philipp Scherer, Albert Einstein College of Medicine) using oligonucleotide primers corresponding to the 5’ (5’-GACGGATCCAAAGCGATGGCGGCGGAG-3’ and 3’ (5’-GACGGATCCAAAGCGATGGCGGCGGAG-3’)) ends of the open reading frame of murine CGI-58 with added BamHI and SalI restriction sites, respectively, using the Expand High Fidelity PCR System (Roche Applied Science). The CGI-58 cDNA was subcloned into pSMT3, a bacterial expression vector encoding a 6-histidine tag and a SUMO moiety containing a Ulp1 cleavage site (28) 5’ to the BamHI and SalI restriction sites, and in-frame with the CGI-58 coding sequence. The subcloned CGI-58 cDNA was sequenced to confirm fidelity of amplification. Bacteria expressing recombinant CGI-58 were solubilized in 500 mM sodium chloride, 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 10 mM octanoyl-N-methylglycamide (Mega-8; Anastrace, Maumee, OH), 10 mM Tris, pH 7.8 (Solution 1) with 5 mM imidazole, by probe sonication for 10 min on ice (15 a pulses every 45 s) followed by centrifugation in a Beckman Type 45 Ti rotor at 150,000 \(x\) g for 1 h. Supernatant was eluted over Ni-NTA agarose resin (Qiagen) packed into a XK16 low pressure FPLC column (Amersham Biosciences) with 250 mM imidazole in Solution 1. The eluted protein containing the recombinant CGI-58 was dialyzed against Solution 2 with continuous cleavage of Ulp1 protease (100 \(\mu\)g/ml) overnight at 4 °C, prior to elution three times over BD Talon Superflow Cobalt resin in Solution 1. Proteins were dialyzed against 150 mM sodium chloride, 1 mM TCEP, 10 mM Mega-8, 10 mM Tris, pH 7.8 (Solution 2) prior to elution over a Mono Q HR 10/10 anion exchange column (Amersham Biosciences) with 1 M sodium chloride, 1 mM TCEP, 10 mM Mega-8, 10 mM Tris, pH 7.8. Fractions containing recombinant CGI-58 were then dialyzed against Solution 2 prior to elution over a Superdex 75 column (Amersham Biosciences) in Solution 2. A single protein band of ~39 kDa was observed on Coomassie Blue-stained gels from SDS-PAGE; this protein was used to immunize two rabbits to raise polyclonal antisera against murine CGI-58 (Covance Research Products, Denver, PA).

**Stable Expression of CGI-58-GFP Chimeras in 3T3-L1 Cells**—A murine CGI-58 cDNA was amplified out of the 3T3-L1 adipocyte library using oligonucleotide primers corresponding to the 5’-coding sequence lacking the first 6 amino acids (5’-GCTTCAGAGGAGGTGGACTCGGAG-3’) and 3’-coding and non-coding sequences including the stop codon (5’-GCTTCAGAGGAGGTGGACTCGGAG-3’) with added XbaI restriction sites using the Expand High Fidelity PCR System (Roche Applied Science). The CGI-58 cDNA was subcloned into the unique XbaI site of the pSREsMVsKneo-GFP retroviral expression vector (29) to express CGI-58 with an N-terminal fused GFP. An additional GFP-CGI-58 fusion retroviral expression construct was produced using ampicillin resistance from the entire coding sequence of CGI-58, including the first six amino acids using 5’-GACTTCTCGAGTGAAGCTTGTGGATCTCGAG-3’ and 3’-GACTTCTCGAGTGAAGCTTGTGGATCTCGAG-3’ oligonucleotide primers containing XhoI and BamHI restriction sites, respectively, the full-length CGI-58 cDNA in the pSMT3 vector as a template, and \(Pfu\) polymerase, and subcloning the product into the pLEGFP-C1 retroviral expression vector (Clontech, BD BioSciences), to express CGI-58 with an N-terminal fused GFP. Fidelity of amplification was confirmed by sequencing. Ecotropic retroviral stocks were produced in transfected 293T cells as described previously (26), and used to infect cultured 3T3-L1 preadipocytes. Cells stably expressing CGI-58-GFP or GFP alone were selected for by resistance to gene-Select from Clontech, BD Biosciences.

**Stable Expression of Intact Perilipin A and Mutated Perilipins in 3T3-L1 Preadipocytes**—Cells stably expressing intact perilipin A (26), or mutated perilipins lacking portions of the N and C termini were prepared previously (12, 13); these cell lines include cells stably expressing perilipin mutation N1 (12) (also called mutation N3; Ref. 13) lacking N-terminal sequences from amino acids 1–81, perilipin mutation N4 (12) (also called mutation N7; Ref. 13) lacking N-terminal sequences from amino acids 1 to 222, perilipin mutation C1 (12, 13) lacking C-terminal amino acids 490–517, and perilipin mutation C2 (12, 13) lacking C-terminal amino acids 430–517.

The perilipin B cDNA subcloned into the pSREsMVsKneo retroviral expression vector may specifically be used by Dr. John Tansey (Otterbein College, Westerville, OH). Ecotropic retroviral stocks were produced in transfected 293T cells as described previously (26), and used to infect cultured 3T3-L1 preadipocytes.

Two forms of mutated perilipin A lacking sequences within the C terminus were prepared by replacing the cDNA sequences encoding intact or mutated amino acids 382–429 (21) with portions of the CGI-58 N-terminal restriction site, using the procedure described previously (12); these mutated forms of perilipin were designated C1a and C2a, respectively. The mutated perilipin cDNAs were ligated into the unique HindIII site of the pSREsMVsKneo retroviral expression vector (12, 29). Ecotropic retroviral stocks were produced in transfected 293T cells as described previously (26), and used to infect cells stably expressing CGI-58, while stably expressing the CGI-58 cDNA were selected for by resistance to gene-Select at 0.6 mg/ml culture medium.

**Immunofluorescence Microscopy**—3T3-L1 preadipocytes expressing intact or mutated perilipins, or lacking ectopic perilipins were cultured...
on glass-cover slips for microscopy experiments. For experiments examining CGI-58 localization in 3T3-L1 preadipocytes, cells were incubated overnight with 400 μM oleic acid complexed to bovine serum albumin (26) to increase the synthesis and storage of triacylglycerol in lipid droplets. Some 3T3-L1 preadipocytes were incubated with 10 μM forskolin and 0.5 mM IBMX for 1 h to elevate cAMP levels and activate PKA. Some differentiated 3T3-L1 adipocytes were incubated with 10 μM isoprotrenol and 0.5 mM IBMX to elevate cAMP levels. To subsequently inhibit the β-adrenergic signaling pathway, adipocytes were rinsed with phosphate-buffered saline, and fresh culture medium containing 200 μM propanolol was added for an additional 30 min to 4 h. Cells were fixed in 3% paraformaldehyde in phosphate-buffered saline and processed further, as described previously (12). Antibodies used were rabbit polyclonal anti-GFP sera diluted 1:250, rabbit polyclonal anti-CGI-58 sera diluted 1:1500, and rabbit polyclonal antisera raised against a fusion protein of the perilipin N terminus (6) diluted 1:1500, or guinea pig polyclonal antisera raised against a fusion protein of the perilipin C terminus (6) diluted 1:1000. Secondary antibody staining was accomplished with Alexa Fluor 546 goat anti-rabbit IgG or rhodamine-conjugated goat anti-rabbit IgG following the manufacturer’s recommendations. In some experiments, fixed cells were co-stained with Bodipy 493/503 during the secondary antibody incubation (26). Cells were viewed with a Nikon Eclipse E800 fluorescence microscope equipped with either a Hamamatsu Orca digital camera interfaced with a Power Macintosh G4 computer or an Optronics MagnaFire digital camera interfaced with a Dell Dimension XPS Pentium II computer. Images were acquired in monochrome and processed to color images using either ImagePro Openlab software or Optronics MagnaFire software; cells co-stained with rhodamine or Alexa-Fluor 546 secondary antibodies and Bodipy 493/503 are depicted in the opposite colors for esthetic purposes.

Northern Blot Analysis—Nitrocellulose membranes containing poly(A) mRNA from mouse tissues (OriGene Technologies, Inc., Rockville, MD and BioChain Institute, Inc., Hayward, CA) were probed with 32P-labeled cDNA probes for murine CGI-58.

Immunoblotting—Proteins in whole cell homogenates or isolated lipid droplet fractions (12) from 3T3-L1 preadipocytes or 3T3-L1 adipocytes differentiated for 10 days (6) were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes; cellular protein content was measured by the bicinchoninic acid method (30) to adjust protein load. Membranes were probed with either polyclonal antisera raised against recombinant CGI-58 diluted 1:25,000, or polyclonal antisera raised against recombinant peptides of perilipin A (6) diluted 1:25,000 followed by a horseradish peroxidase-conjugated secondary antibody or horseradish peroxidase-conjugated secondary antibody; enhanced chemiluminescence reagents (Amersham Biosciences) were used for signal detection.

Immunoprecipitations—3T3-L1 preadipocytes stably expressing ectopic perilipin A, perilipin B, or mutated forms of perilipin A were lipid-loaded overnight with 400 μM oleic acid complexed to albumin prior to 1 h incubation in the presence or absence of 10 μM forskolin and 0.5 mM IBMX. Cells were lysed in 150 mM NaCl, 1% Triton X-100, 60 mM N-Octylglucoside, 10 mM Tris, pH 8.0 supplemented with protease inhibitors (Roche Applied Science) (IP Solution) for 15 min at 4 °C; positive control samples were lysed 3T3-L1 adipocytes differentiated for 10 days (6). Lysates were cleared by centrifugation at 14,000 × g for 10 min at 4 °C, and preincubated with protein A-Sepharose prior to the immunoprecipitation protocol. Supernatants were incubated with polyclonal antibodies raised in rabbits against a recombinant N-terminal peptide of perilipin A (6) or control non-immune rabbit serum, and protein A-Sepharose at 4 °C overnight, washed extensively in IP solution, and then eluted in Laemmli sample buffer prior to SDS-PAGE and immunoblotting.

RESULTS

CGI-58 mRNA Is Most Highly Expressed in Adipose Tissue and Testes—We initially identified the mouse homolog of human CGI-58 as a protein component of lipid droplets isolated from cultured 3T3-L1 adipocytes using protein mass spectrometry analysis. To develop reagents to confirm the localization of CGI-58 to lipid droplets and to study CGI-58 function, we amplified the murine CGI-58 cDNA out of a bacterial library (6) using primers designed to amplify the small protein SUMO. Following removal of the SUMO tag fusion protein with an N-terminal 6-histidine tag followed by affinity chromatography, Mouse CGI-58 was expressed in bacteria as a recombinant small protein. Recombinant CGI-58 was 6-fold higher in 3T3-L1 adipocytes indicating that CGI-58 was 6-fold higher in 3T3-L1 adipocytes indicating that CGI-58 expression is induced during adipocyte differentiation. Endogenous CGI-58 from cultured mammalian cells migrates on SDS-PAGE gels with an apparent molecular weight of 6 kDa larger than the predicted molecular weight of 39 kDa. Other lipid droplet-associated proteins such as perilipin A show a similar shift in migration suggesting that the retarded mobility may be a common property of lipid droplet-associated proteins; alternatively, CGI-58 may be post-translationally modified in mammalian cells. A PROSITE pattern search of the predicted amino acid sequence of mouse CGI-58 revealed a consensus sequence for phosphorylation by PKA, and multiple consensus sequences for phosphorylation by protein kinase C and casein kinase 2. While potential sites for myristoylation were also

![Image](https://example.com/figure.png)
Noted, we eliminated possible myristoylation of the protein since we have evidence that we present in this study that GFP fused to the N terminus is retained with CGI-58; furthermore, the localization properties of CGI-58-GFP match those of endogenous CGI-58.

**CGI-58 Localizes to Cytosol in 3T3-L1 Preadipocytes and to the Surfaces of Lipid Droplets in 3T3-L1 Adipocytes.—**To study the localization of CGI-58 in cultured cells, we stably expressed CGI-58 as a fusion protein with GFP appended to its N terminus in 3T3-L1 preadipocytes. By immunofluorescence microscopy, CGI-58-GFP displayed a diffuse distribution throughout the cytoplasm of 3T3-L1 preadipocytes, with a very low level of staining on the surfaces of lipid droplets (Fig. 3A). We tested the possibility that the localization of CGI-58-GFP to lipid droplets may require the activation of PKA in a mechanism comparable to the translocation of PKA-phosphorylated hormone-sensitive lipase from the cytosol to the surfaces of lipid droplets in adipocytes (17, 31–33). Treatment of 3T3-L1 preadipocytes stably expressing CGI-58-GFP with forskolin and IBMX to elevate cAMP and activate PKA failed to alter the diffuse distribution of CGI-58-GFP (Fig. 3B). The cytoplasmic localization of CGI-58 was unexpected following our identification of CGI-58 on lipid droplets isolated from 3T3-L1 adipocytes by mass spectrometry, hence, we next asked whether CGI-58-GFP localizes to lipid droplets in adipocytes. 3T3-L1 preadipocytes stably expressing CGI-58-GFP were grown to confluence and induced to differentiate into adipocytes. By immunofluorescence microscopy, CGI-58-GFP showed clear localization to the surfaces of lipid droplets in fully differentiated adipocytes (Fig. 3C), while GFP retained a diffuse distribution throughout the cytoplasm (Fig. 3D). Identical results were obtained with CGI-58-GFP chimeras including the entire coding sequence of CGI-58, and CGI-58 lacking the first 6 amino acids (Met-Lys-Ala-Met-Ala-Ala), suggesting that the initial 6 amino acids of CGI-58 are not required to target CGI-58-GFP to lipid droplets in adipocytes.

Production of high titer polyclonal antisera raised against recombinant CGI-58 facilitated the detection of endogenous CGI-58 in cells and confirmation of the subcellular localization results obtained with studies of CGI-58-GFP chimeras. By immunofluorescence microscopy, endogenous CGI-58 showed a diffuse distribution in 3T3-L1 preadipocytes (Fig. 3E), but clear localization to the surfaces of lipid droplets in differentiated adipocytes (Fig. 3F). Thus, localization of the CGI-58-GFP chimera mirrored the localization of endogenous CGI-58. CGI-58 localizes to the surfaces of lipid droplets in differentiated 3T3-L1 adipocytes, but primarily to the cytoplasm in preadipocytes.

**CGI-58 Disperses from Lipid Droplets When PKA Is Activated in 3T3-L1 Adipocytes.—**CGI-58 localized to the surfaces of lipid droplets in adipocytes under normal culture conditions (basal conditions) when the storage of triacylglycerol predominates and the basal rate of lipolysis is low. Because CGI-58 is a member of the α/β-hydrolase fold family of proteins that includes many lipases, we were interested in studying the localization of CGI-58 under conditions when lipolysis is stimulated and hormone-sensitive lipase translocates from the cytosol to the surfaces of lipid droplets (17, 31–33). Fully differentiated 3T3-L1 adipocytes stably expressing ectopic CGI-58-GFP were treated with isoproterenol to stimulate β-adrenergic receptor signaling, and IBMX to inhibit phosphodiesterase activity and preserve elevated levels of cAMP. Strikingly, CGI-58 binding to adipocyte lipid droplets.
CGI-58 Binding to Adipocyte Lipid Droplets

Figure 4. CGI-58-GFP reversibly disperses off of lipid droplets when 3T3-L1 adipocytes are treated with isoproterenol and IBMX to stimulate lipolysis. Differentiated 3T3-L1 adipocytes stably expressing CGI-58-GFP were either fixed with paraformaldehyde (A), or treated with 10 μM isoproterenol and 0.5 mM IBMX to elevate cellular cAMP levels and activate PKA for 5 min (B), 15 min (C), or 30 min (D) prior to fixation. Adipocytes in E were incubated with isoproterenol and IBMX for 2 h before removing the culture medium and adding fresh culture medium containing 200 μM propranolol for 30 min prior to fixation. F, adipocytes stably expressing GFP were treated with isoproterenol and IBMX for 4 h prior to fixation. All cells were stained with polyclonal antibodies against GFP (green) and Bodipy 493/503 (red).

58-GFP rapidly departed from the surfaces of lipid droplets, and dispersed throughout the cytoplasm of the cells (Fig. 4). Significant displacement of CGI-58-GFP from lipid droplet surfaces was observed within 5 min (Fig. 4B), and the redistribution of CGI-58-GFP into the cytosol was essentially complete by 30 min (Fig. 4D); a very low level of CGI-58-GFP staining was observed at lipid droplet surfaces at all incubation times up to and including 4 h of treatment with isoproterenol and IBMX (not shown). Moreover, the redistribution of CGI-58-GFP from the surfaces of lipid droplets to the cytosol was reversible following the removal of isoproterenol and IBMX from the culture medium and the addition of 200 μM propranolol, a β-adrenergic receptor antagonist. CGI-58-GFP returned to the surfaces of lipid droplets within a 30-min period following the addition of propranolol (Fig. 4E). By contrast, GFP retained a diffuse distribution in the cytoplasm during the treatment of 3T3-L1 adipocytes stably expressing GFP with isoproterenol and IBMX for up to 4 h (Fig. 4F). Similar results were obtained in differentiated 3T3-L1 adipocytes visualized with antibodies raised against CGI-58 to detect the endogenous protein (data not shown). Interestingly, the timing of CGI-58 dispersion off of lipid droplets appears to be coordinated with the opposite translocation of HSL from the cytoplasm to the surfaces of lipid droplets, and the initiation of stimulated lipolysis, which occurs after a 5-min delay (31).

CGI-58 Co-localizes with Perilipin A at the Surfaces of Lipid Droplets in 3T3-L1 Preadipocytes Stably Expressing Ectopic Perilipin A—CGI-58-GFP and endogenous CGI-58 localize to the surfaces of lipid droplets in differentiated 3T3-L1 adipocytes, but display a diffuse distribution throughout the cytosol in preadipocytes. Adipophilin is the major structural protein coating the lipid droplets of 3T3-L1 preadipocytes prior to the induction of perilipin expression during adipocyte differentiation. Perilipin A displaces adipophilin from the surfaces of the enlarging lipid droplets of adipocytes ~3 days after the initiation of differentiation (6). Therefore, we asked whether CGI-58 localizes to adipocyte lipid droplets because of the surface coating of perilipins. To address this question, we tested whether endogenous CGI-58 localizes to the surfaces of lipid droplets in 3T3-L1 preadipocytes stably expressing ectopic perilipin A. Anti-CGI-58 antibodies decorated the surfaces of lipid droplets in 3T3-L1 preadipocytes expressing ectopic perilipin A (Fig. 5A) but not in control cells stably expressing the retroviral expression vector but lacking perilipins (Fig. 5D). More than 85% of cells expressing ectopic perilipin A showed clear localization of CGI-58 to lipid droplet surfaces (Table I), while 0% of control cells lacking perilipins showed significant localization of CGI-58 to lipid droplets, but instead, showed diffuse distribution of CGI-58 throughout the cytoplasm. The co-localization of staining for CGI-58 and perilipin on lipid droplets in cells expressing perilipin A (Fig. 5C), but not control cells (Fig. 5F), suggests that the two proteins are in very close proximity at the surfaces of lipid droplets and that perilipin A is required for the localization of CGI-58 to lipid droplets.

Endogenous CGI-58 Binds to the Surfaces of Lipid Droplets Covered with Intact Perilipin A, or Mutated Perilipin A Lacking N-terminal Sequences, but Not Mutated Perilipin Lacking C-terminal Sequences.—We used a collection of cell lines of 3T3-L1 preadipocytes stably expressing various mutated forms of perilipin A (12, 13) to identify the sequence of perilipin critical for CGI-58 binding; the cells were stained with polyclonal antisera raised against CGI-58 to detect endogenous CGI-58. Mutated forms of perilipin used in this study were previously shown to target to lipid droplets (12, 13). CGI-58 localized to the surfaces of lipid droplets in cells stably expressing intact perilipin A (Fig. 6, Peri A, Basal and Table I), or two truncation mutations of perilipin A lacking either the first 81 amino acids (Fig. 6, N1, Basal) or the first 222 amino acids (Fig. 6, N4, Basal) of the N terminus. In contrast, CGI-58 failed to localize to lipid droplets coated with mutated perilipin lacking the final 173 amino acids of the C terminus (Fig. 6, C5, Basal), or to control cells lacking perilipins (Fig. 6, Control, Basal), but instead, retained a diffuse distribution throughout the cytoplasm.

The 3T3-L1 preadipocytes stably expressing perilipin A and mutated forms of perilipin were incubated with 10 μM forskolin and 0.5 mM IBMX to elevate cAMP levels, activate PKA, and promote phosphorylation of the ectopic perilipin. CGI-58 dispersed from the surfaces of lipid droplets coated with ectopic perilipin A, as it does in 3T3-L1 adipocytes, following the activation of PKA (Fig. 6, Peri A, +forskolin/IBMX); 89% of cells showed complete dispersion of CGI-58, while 11% of cells showed partial dispersion of CGI-58 into the cytoplasm with a low level of residual staining of lipid droplets (Table I). Furthermore, CGI-58 dispersed from lipid droplets in cells stably expressing the N1 mutation. In contrast, CGI-58 maintained a diffuse distribution in 3T3-L1 preadipocytes stably expressing perilipin mutation C5 and in control cells lacking perilipins following treatment with forskolin and IBMX.

To further map the C-terminal sequence of perilipin A that mediates CGI-58 association with lipid droplets, we tested the localization of endogenous CGI-58 in 3T3-L1 preadipocytes stably expressing additional mutated forms of perilipin A that lack portions of the C terminus. CGI-58 localized to lipid droplets...
amino acid sequence; perilipin B shares 405 amino acids with perilipin A followed by a unique C-terminal sequence of 17 amino acids.

complete dispersion of CGI-58 throughout the cytoplasm. Perilipin A contains a unique C-terminal sequence of 112 amino acids within its 517 amino acids.

CGI-58, viewed with a fluorescence microscope, and scored for the percent of cells displaying strong localization of CGI-58 to lipid droplets or complete dispersion of CGI-58 into the cytoplasm. Perilipin A contains a unique C-terminal sequence of 112 amino acids within its 517 amino acid sequence; perilipin B shares 405 amino acids with perilipin A followed by a unique C-terminal sequence of 17 amino acids.

covered with mutated perilipin that lacks the final 28 amino acids of the C terminus including PKA sites 5 and 6 (Fig. 7A, C1). Similarly, CGI-58 localized to lipid droplets covered with mutated perilipin that lacks the final 88 amino acids of the C terminus including PKA sites 4, 5, and 6 (Fig. 7A, C2). Staining for CGI-58 was observed on lipid droplets in 93 and 86% of cells expressing perilipin mutations C1 and C2, respectively (Table I). CGI-58 failed to significantly localize to lipid droplets covered with mutated perilipin lacking either amino acids 382–405 in the C terminus (Fig. 7A, C1) or amino acids 406–429 (Fig. 7A, C2). The sequences of perilipin isoforms A and B are identical from amino acids 1 through 405, but diverge in the C termini. The identification of a docking site for CGI-58 between amino acids 382 and 429 of perilipin A suggests that CGI-58 may not localize to lipid droplets coated with perilipin B. As predicted, CGI-58 failed to localize to lipid droplets in 3T3-L1 preadipocytes stably expressing ectopic perilipin B (Fig. 7A, Peri B). Further mutagenesis of the sequence between amino acids 382 and 429 of perilipin A including deletion of amino acids 382–397, amino acids 398–413, or amino acids 414–429 failed to home in on a shorter sequence for CGI-58 docking; endogenous CGI-58 showed a diffuse cytoplasmic distribution in cells expressing each of these mutated forms of perilipin (data not shown).

The cell lines stably expressing mutated forms of perilipin A with deletions of C-terminal sequences were incubated with forskolin and IBMX to activate PKA, and the localization of endogenous CGI-58 was assessed. Following the activation of PKA, 99% of cells expressing intact perilipin A showed dispersion of CGI-58 into the cytoplasm (Table I). In contrast, 79 and 21% of cells expressing perilipin mutation C1 (lacking PKA sites 5 and 6) showed full and partial dispersion of CGI-58 into the cytoplasm, respectively, while 58 and 42% of cells expressing perilipin mutation C2 (lacking PKA sites 4, 5, and 6) showed full and partial dispersion of CGI-58 into the cytoplasm, respectively (Table I). Thus, CGI-58 clearly localized to lipid droplets coated with perilipin mutation C2, but failed to fully disperse into the cytoplasm following the activation of PKA. These results suggest that the phosphorylation of serines in the C-terminal PKA consensus sites of perilipin A as well as PKA consensus site(s) of CGI-58 may be critical for the translocation of CGI-58 away from lipid droplets and into the cytoplasm.

The cell lines displaying stable expression of perilipin mutations C1 and C2 have been characterized previously (12, 13), however, we have not previously reported the cell lines with stable expression of perilipin mutations C3, C4, and C5, or perilipin B. Therefore, the localization of perilipin B and the mutated forms of perilipin to lipid droplets was characterized by

| Form of perilipin | Amino acids expressed | Full or partial localization of CGI-58 to lipid droplets<sup>a</sup> | % of cells |
|------------------|----------------------|-----------------------------|------------|
| Perilipin A      | 1–517/112 unique     | 85                          | 11<sup>b</sup> |
| Perilipin B      | 1–422/17 unique      | 4<sup>a</sup>               | 1<sup>b</sup> |
| Mutation N1      | 82–517               | 86                          | 15<sup>b</sup> |
| Mutation N4      | 223–517              | ND<sup>b</sup>              | ND         |
| Mutation C1      | 1–489                | 93                          | 21<sup>b</sup> |
| Mutation C2      | 1–429                | 86                          | 42<sup>b</sup> |
| Mutation C3Δ1    | 1–381; 406–517       | 12<sup>a</sup>              | 0          |
| Mutation C3Δ2    | 1–405; 430–517       | 8<sup>a</sup>               | 0          |
| Mutation C5      | 1–344                | 3<sup>a</sup>               | 0          |
| Control          | No perilipin         | 0                           | 0          |

<sup>a</sup> Weak association of CGI-58 with lipid droplets and partial dispersion in the cytoplasm.

<sup>b</sup> ND, not determined.
immunofluorescence microscopy (Fig. 7A), and immunoblotting (Fig. 7C); perilipin B and all four forms of mutated perilipin A localized to the surfaces of lipid droplets at levels similar to that of intact ectopic perilipin A.

Co-immunoprecipitation of CGI-58 with Mutated Forms of Perilipin Confirms the Identification of a C-terminal Binding Site for CGI-58—To confirm the observations made using immunofluorescence microscopy, lysates from 3T3-L1 adipocytes or lipid-loaded 3T3-L1 preadipocytes stably expressing intact perilipin A or mutated forms of perilipin were used for immunoprecipitation studies using antibodies raised against a recombinant N-terminal peptide of perilipin A (6). CGI-58 was co-precipitated from lysates of 3T3-L1 adipocytes, and 3T3-L1 preadipocytes expressing ectopic perilipin A, perilipin B, C1-mutated perilipin, and C2-mutated perilipin, but not the C5, CΔ1, and CΔ2 forms of mutated perilipin (Fig. 8). CGI-58 was not co-precipitated with any forms of ectopic perilipin when 3T3-L1 preadipocytes were incubated with forskolin and IBMX, although a small amount of CGI-58 was recovered from immunoprecipitations of perilipin from differentiated 3T3-L1 adipocytes treated with forskolin and IBMX. Neither perilipin nor CGI-58 was precipitated from lysates of 3T3-L1 adipocytes with control non-immune serum.

**DISCUSSION**

The major finding of this study is that CGI-58, a protein that is highly expressed in adipocytes, binds to perilipin A on the surfaces of adipocyte lipid droplets. Remarkably, the localization of CGI-58 to lipid droplets is regulated by the metabolic status of the adipocyte. The incubation of 3T3-L1 adipocytes with isoproterenol, a β-adrenergic agonist, triggers the rapid but reversible translocation of CGI-58 from the surfaces of lipid droplets to the cytoplasm; thus, the phosphorylation of perilipin A, CGI-58, or both proteins by PKA disrupts the binding interaction and releases CGI-58. In contrast, both endogenous CGI-58 and an ectopic CGI-58-GFP chimera are diffusely distributed throughout the cytoplasm of 3T3-L1 preadipocytes, despite the presence of small lipid droplets. Adipophin, a protein that is related to perilipins, coats the surfaces of lipid droplets in preadipocytes, fibroblasts, and many other types of cells, but fails to efficiently bind CGI-58.

We mapped the CGI-58 binding site to a sequence of 48 amino acids between positions 382 and 429 near the C terminus of perilipin A; this sequence spans the junction at amino acid 405 where the sequences of perilipin isoforms A and B diverge. Consistent with these results, CGI-58 failed to localize to lipid droplets coated with perilipin B, suggesting that a portion of the unique C terminus of perilipin A is required for binding. The deletion of short sequences between amino acids 382 and 429 failed to identify a smaller binding site, suggesting that a highly acidic sequence (purple oval), and the end of the sequence common to perilipin A and B at amino acid 405 (brown vertical line) that is followed by a unique C-terminal sequence of 112 amino acids in perilipin A, or 17 amino acids in perilipin B. Six consensus sites for phosphorylation of serines by PKA are spread throughout the sequence (small yellow circles).
that either CGI-58 binds to multiple sequences spread throughout the region, or the intact 48-amino acid sequence is required to fold into a conformation that exposes a smaller binding interface. The data obtained from co-immunoprecipitation experiments were mostly consistent with our findings in cell-based experiments; however, a notable difference was the co-precipitation of CGI-58 with perilipin B. Since perilipin B contains the sequence from amino acid 382 to 405, our results suggest that the position or conformation of a partial docking site for CGI-58 may be altered by the unique 17-amino acid sequence of perilipin B (406–422) when perilipin is associated with lipid droplets, but exposed when cells are lysed, and the proteins are solubilized away from lipid droplets.

In adipocytes, extensive lipolysis is triggered when catecholamines bind to cell surface β-adrenergic receptors initiating a signaling cascade that elevates cAMP levels and activates PKA. Perilipin A becomes phosphorylated on as many as six serines facilitating the docking of PKA-phosphorylated hormone-sensitive lipase on lipid droplets (17) and the hydrolysis of triacylglycerol. Interestingly, CGI-58 translocates off of adipocyte lipid droplets and disperses throughout the cytosol when lipolysis is stimulated, suggesting that CGI-58 may play a role in lipid metabolism either when it is bound to lipid droplets under basal conditions, or during its movement away from lipid droplets following activation of PKA. While the phosphorylation of PKA sites 1 and 2 of perilipin A is unnecessary for the displacement of CGI-58 from lipid droplets, mutations altering PKA sites 4, 5, and 6 attenuate dispersion. Since CGI-58 contains at least one PKA consensus site, it is likely that the phosphorylation of serines in PKA consensus sites of both perilipins is involved in the regulation of CGI-58 binding to lipid droplets.
CGI-58 Binding to Adipocyte Lipid Droplets

FIG. 8. Co-immunoprecipitation of CGI-58 with perilipin A and mutated forms of perilipin. Lysates were prepared from lipid-loaded 3T3-L1 preadipocytes stably expressing ectopic perilipin A (PeriA), perilipin B (PeriB), mutated forms of perilipin A (C1, C2, C5, CA1, C32), or lacking ectopic perilipin (Con), and 3T3-L1 adipocytes after 10 days of differentiation (Day 10) treated for 1 h with or without 10 μM forskolin and 0.5 mM IBMX prior to immunoprecipitation (IP) with anti-perilipin antisera followed by SDS-PAGE and immunoblotting (IB) with either anti-CGI-58 or anti-perlipin antisera. The two lanes to the right of the vertical line are immunoprecipitations of Day 10 adipocyte lysates with control non-immune rabbit serum followed by immunoblotting with either anti-CGI-58 or anti-perlipin antisera. Immunoprecipitations depicted are from a representative experiment out of three. The characteristic shift in apparent molecular weight of perilipin A following forskolin/IBMX treatment is more pronounced in forms of perilipin with an intact C terminus.

CGI-58 and perilipin A are required for the full dispersion of CGI-58 into the cytoplasm. Thus, the perilipin-dependent binding of CGI-58 to lipid droplets is modulated by the activation of PKA. We hypothesize that perilipins form a scaffold at the surfaces of adipocyte lipid droplets that serves as an organizing center for lipid metabolic enzymes and transporters that is altered by metabolic signals. While proteins including CGI-58 bind to perilipin-coated lipid droplets under basal conditions, other proteins, such as hormone-sensitive lipase, are recruited to lipid droplets when perilipins have been phosphorylated by PKA. Under the latter conditions, protein components of basal lipid droplets may be displaced to accommodate association of the lipolytic machinery.

What role then, does CGI-58 play in lipid metabolism? CGI-58 is a member of the α/β hydrolase fold subfamily of lipases, esterases, and thioesterases. Mutations of human CGI-58 are responsible for excessive triacylglycerol storage in many types of cells (23–25). Studies of triacylglycerol metabolism in fibroblasts from NLSD patients show that CGI-58 is required for normal rates of triacylglycerol turnover (34–36); however, triacylglycerol lipase activity in NLSD cell lysates was comparable to that of control cells (34). Additionally, the rate of triacylglycerol clearance increased in NLSD cells incubated with triacsin C, an inhibitor of acyl CoA synthetases, although not quite to the higher rates observed in control cells (35). These observations suggest that lipolysis occurs in NLSD cells, but that triacylglycerol clearance is blunted by rapid re-esterification (35). Thus, CGI-58 is a factor that is required to assist triacylglycerol clearance, but may not be a lipase.

Two tissues with high levels of expression of CGI-58 are adipose tissue and testes; these tissues are among the few that express perilipins. Although the high levels of CGI-58 in adipocytes imply an important role for the protein in adipocyte triacylglycerol metabolism, there have been no reports of obesity in humans with NLSD. Furthermore, CGI-58 shows a subcellular localization pattern that is paradoxically opposite to that of hormone-sensitive lipase; CGI-58 disperses off of lipid droplets when hormone-sensitive lipase translocates onto the droplets, and the rate of lipolysis becomes elevated. Interestingly, basal rates of lipolysis are relatively normal in adipocytes isolated from HSL null mice (37–39), suggesting that as yet uncharacterized mechanisms catalyze triacylglycerol turnover under basal conditions. Thus, CGI-58 may interact with perilipin-coated lipid droplets to play a role in basal triacylglycerol hydrolysis. Alternatively, CGI-58 may function to transport products of lipolysis away from lipid droplets following the activation of PKA and the dispersion of CGI-58 into the cytosol.

Since adipose triacylglycerol metabolism appears not to be severely compromised in NLSD patients, it is likely that adipocytes have redundant mechanisms to avoid the detrimental effects of mutations in CGI-58. Thus, despite much lower levels of expression, CGI-58 may play a more critical role in triacylglycerol catabolism in non-adipose tissues.

Other researchers have identified CGI-58 as a component of lipid droplets isolated from oleic acid-treated Chinese hamster ovary cells (20) and cultured human epithelial cells (21), cells that lack perilipins but instead, coat lipid droplets with adipophillin. While this article was being prepared, a paper appeared in press (40) that identified CGI-58 as a perilipin-binding protein by yeast two-hybrid assay using the N-terminal sequence of perilipin from amino acid 1 to 250 as bait. These researchers also demonstrated direct binding between recombinant CGI-58 and recombinant truncated perilipin A that included amino acids 1–416. In contrast, we have found that CGI-58 binding to lipid droplets in intact cells requires sequences in the C terminus of perilipin A, but not the N terminus. These investigators further characterized a binding interaction between CGI-58 and adipophillin by yeast two-hybrid and GST pull-down assays using recombinant proteins. In contrast to the reported results, we did not observe significant localization of CGI-58 to lipid droplets in 3T3-L1 preadipocytes, where adipophillin is the predominant structural protein of lipid droplets. Furthermore, the PAT family of proteins is highly conserved within N-terminal sequences, but structurally divergent in the C termini, where we have identified a binding site for CGI-58 on perilipin A. Finally, while both studies show that CGI-58-GFP chimeras localize to lipid droplets in differentiated 3T3-L1 adipocytes, we have found that the perilipin-dependent association of CGI-58 with lipid droplets is regulated by the metabolic state of the adipocyte.

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