Structure of a CGI-58 Motif Provides the Molecular Basis of Lipid Droplet Anchoring

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Background: CGI-58 activates the key intracellular lipase ATGL.

Results: Solution structure of the N-terminal lipid droplet (LD)-binding motif of CGI-58 bound to dodecylphosphocholine micelles.

Conclusion: The LD-binding motif acts independently to anchor proteins to LDs and consists of two LD-binding arms.

Significance: The structure of the peptide LD anchor sheds light on the interaction of CGI-58 with LDs.

Triacylglycerols (TGs) stored in lipid droplets (LDs) are hydrolyzed in a highly regulated metabolic process called lipolysis to free fatty acids that serve as energy substrates for β-oxidation, precursors for membrane lipids and signaling molecules. Comparative gene identification-58 (CGI-58) stimulates the enzymatic activity of adipose triglyceride lipase (ATGL), which catalyzes the hydrolysis of TGs to diacylglycerols and free fatty acids. In adipose tissue, protein-protein interactions between CGI-58 and the LD coating protein perilipin 1 restrain the ability of CGI-58 to activate ATGL under basal conditions. Phosphorylation of perilipin 1 disrupts these interactions and mobilizes CGI-58 for the activation of ATGL. We have previously demonstrated that the removal of a peptide at the N terminus (residues 10–31) of CGI-58 abrogates CGI-58 localization to LDs and CGI-58-mediated activation of ATGL. Here, we show that this tryptophan-rich N-terminal peptide serves as an independent LD anchor, with its three tryptophans serving as focal points of the left (harboring Trp21 and Trp25) and right (harboring Trp29) anchor arms. The solution state NMR structure of a peptide comprising the LD anchor bound to dodecylphosphocholine micelles as LD mimic reveals that the left arm forms a concise hydrophobic core comprising tryptophans Trp21 and Trp25 and two adjacent leucines. Trp29 serves as the core of a functionally independent anchor arm. Consequently, simultaneous tryptophan alanine permutations in both arms abolish localization and activity of CGI-58 as opposed to tryptophan substitutions that occur in only one arm.

Triacylglycerols (TGs)2 are stored in lipid droplets (LDs) comprising a core of neutral lipids (TGs and sterol esters) surrounded by a monolayer of phospholipids (1). The protein “comparative gene identification 58 (CGI-58),” also known as α/β-hydrolase domain 5 (ABHD5), is an important stimulatory protein of the first step in intracellular lipolysis (2, 3). In this catabolic process, adipose triglyceride lipase (ATGL) catalyzes the hydrolysis of TGs stored in LDs to diacylglycerols and free fatty acids (FFAs). Hormone-sensitive lipase and monoacylglycerol lipase subsequently hydrolyze diacylglycerols and monoacylglycerols, respectively, to generate FFAs and glycerol molecules (4).

Mutations in the human gene encoding CGI-58 lead to neutral lipid storage disease with a severe skin defect termed ichthyosis (NLSD-I) (5). Although the ATGL stimulating function of CGI-58 appears causative for the neutral lipid storage phenotype in affected patients, the frequently observed symptoms of hepatomegaly, hepatic steatosis, and ichthyosis are indicative of an ATGL-independent function of CGI-58 (6–8).

The rate of intracellular lipolysis on the surface of LDs depends on post-translational modification events, multiple protein-protein interactions and lipase-ligand interactions at the lipid-water interphase (2, 9–23). Under basal conditions, CGI-58 binds to the LD coating protein perilipin 1 in 3T3-L1 adipocytes (20). In this state, CGI-58 does not interact with ATGL and ATGL activity remains low (24, 25). Phosphorylation of perilipin 1 and CGI-58 by protein kinase A (PKA) leads to rapid release of CGI-58 from perilipin 1-coated LDs and subsequent activation of ATGL (18, 26).

The role of perilipin 1 in the recruitment of CGI-58 to LDs is not well understood. CGI-58 has been shown to activate ATGL also on artificial LD substrates lacking perilipins (12, 15). Currently, it remains unknown whether CGI-58-mediated activa-

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tion of lipolysis occurs due to increased access to the substrate, conformational changes induced in ATGL, increased product release (e.g., channeling the produced FFA away from the reaction site), or increased lipolysis due to interaction with fatty acid-binding proteins (16). Interestingly, the selectivity of ATGL hydrolysis at the sn-2 position of the glycerol backbone broadens to the sn-1 position upon interaction with CGI-58 (27). The lack of high-resolution structures of CGI-58 and ATGL or the protein-protein complex in the presence of a LD surface represents a major bottleneck in understanding the function of these proteins and their interaction surfaces.

The interaction of CGI-58 with ATGL occurs within the N-terminal patatin domain-related region of ATGL (12, 28). A homology model of CGI-58 reveals a core α/β-hydrolase structure consisting of eight mostly parallel β-strands surrounded by α-helices and loops, a cap region comprising α-helices, and a short mostly unstructured N-terminal tryptophan-rich stretch (15). Almost the entire CGI-58 protein is required to activate ATGL, because CGI-58 variants with major deletions from the N or C terminus are not capable of activating ATGL (15). The N-terminal Trp-rich region serves an essential role in the localization of CGI-58 to LDs, which remains a strict requirement for ATGL activation (15).

To better understand the mechanism of CGI-58 LD binding, we solved the structure of the N-terminal fragment of CGI-58 (peptide Val10 to Lys43, CGI-58_V10-K43) bound to dodecylphosphocholine (DPC) micelles, which serve as excellent mimics of the LD surface. The structure reveals that the region Ser19-Cys30 constitutes a LD anchor motif with Trp21 and Trp25 forming a hydrophobic core along with the hydrophobic residues Leu22 and Leu26. This hydrophobic core constitutes the left arm of the anchor. The more isolated Trp29 is flanked by two prolines (Pro27 and Pro31) and serves as the functionally independent right arm of the anchor.

A fusion protein containing just the CGI-58 LD anchor motif (amino acids 19–35) fused to yellow fluorescent protein (YFP) localizes to LDs supporting the concept of the LD-anchor as an independent functional motif. Selective permutations converting single tryptophans of the LD anchor to alanines do not alleviate the ability of CGI-58 to localize to LDs or activate ATGL. However, substitutions in both arms of the LD anchor (W21A and W29A) abolish the ability of CGI-58 to localize to LDs and to activate ATGL.

**Experimental Procedures**

**Generation of Trp Variants of CGI-58—**Wild type (WT) and all mutants of CGI-58 were cloned into the plasmid pEYFP-N1 (BD-Biosciences Clontech) coding for a C-terminal EYFP tag. Generation of wild type-CGI-58 (WT-CGI-58) and the point-mutated variants W21A, W29A, and W21A/W25A was described earlier (15). The single point mutant W25A and the double mutant W21A/W29A were generated by site-directed mutagenesis of a vector encoding for WT-CGI-58 with a C-terminal EYFP tag. The N-terminal peptide containing just the LD anchor mCGI_19–35 was generated upon amplification of the sequence using the forward primer mCGI_19 YFP N1 forward, 5'-GTGATGACCTAGATGTCAGGATGGCTG-3'; and the reverse primer mCGI_35 YFP N1 reverse, 5'-GGAATAGGATCCGCTGATGAGATGTTGGGACACC-3' followed by ligation into Xhol and BamHI sites of the vector. The correctness of all sequences was verified by DNA sequencing (LGCGenomics, Berlin, Germany).

**Cellular Localization of mCGI-58 Variants—**For localization studies, monkey embryonic kidney cells (COS-7, ATCC CRL-1651) were transfected with expression vectors (pEYFP-N1) encoding WT full-length and point mutants of mouse CGI-58 (mCGI-58) with a C-terminal fusion of YFP. COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) containing 4.5 g/liter of glucose, 10% fetal calf serum (FCS), and penicillin/streptomycin under a humidified atmosphere, 37 °C, and 5% CO2. COS-7 cells were seeded on glass coverslips in 6-well plates (1.2 × 105 cells/well) and transfected with YFP-tagged full-length or mutated mCGI-58 variants. 24 h after transfection, cells were incubated for 20 h in DMEM containing FCS, and supplemented with oleic acid (400 μM) complexed to fatty acid-free BSA (Sigma) in a ratio of 3:1 to increase LD formation. Lysosomes were stained with HCS LipidTOX Red Neutral Lipid stain (Life Technologies) and incubated for 10 min at 37 °C. Microscopy was performed using a Leica TCS SP5 confocal microscope (Leica Microsystems GmbH) with a HCX PL APO CS 63× 1.2 water objective. YFP fluorescence was excited at 514 nm and detected at 522–558 nm. LipidTOX Red was excited at 633 nm and detected at 650–669 nm. Transmission images of cultured cells were also recorded. All presented experiments were repeated independently at least three times.

**Preparation of Cell Extracts for Triglyceride Hydrolase Activity Assay—**Almost all cell lines were transiently transfected with the different CGI-58 clones and pcDNA4/HisMax coding for His-tagged ATGL (28) with Metafectene™ (Biontex GmbH) as described earlier (29). The cells were disrupted by sonication and resuspended in lysis buffer (0.25 M sucrose, 1 mM dithiothreitol, 1 mM EDTA, 20 μg/ml of leupeptin, 2 μg/ml of antipain, 1 μg/ml of pepstatin, pH 7.0). Then, nuclei and unbroken cells were removed by centrifugation at 1000 g for 20 min, and the supernatants were used for triglyceride hydrolase activity assays.

**Assay for Triglyceride Hydrolase Activity—**The substrate for the triglyceride hydrolase assay was prepared as described previously with minor modifications (29). Briefly, triolein and [9,10-3H]triolein (10 μCi/ml) were emulsified in the presence of phosphatidylcholine/phosphatidylinositol using a sonicator (Vinsonic 475, Virtis, Gardiner, NJ) and adjusted to 2.5% BSA (FFA free). The final substrate concentration was 0.3 μmol/ml of triolein and 0.15 mg/ml of phosphatidylcholine/phosphatidylinositol (3:1). The reaction mixture was prepared of lysates containing overexpressed HisMax-mATGL (30 μg total protein) and the lysates expressing the different variants of CGI-58 (30 μg of total protein). Activity assays were performed using 0.1 ml of cell lysate mixture and 0.1 ml of substrate in a water bath at 37 °C for 60 min. The reaction was terminated by adding 3.25 ml of methanol/chloroform/heptane (10:9:7) and 1 ml of 0.1 M potassium carbonate, 0.1 M boric acid, pH 10.5. After centrifugation at 800 g for 20 min, the radioactivity in 0.2 ml of the upper phase was determined by liquid scintillation counting.
Statistical Analysis—TG hydrolase activity measurements were performed in triplicates. Measured activities are represented as mean ± S.D. Statistical significance was determined by the Student’s unpaired two-tailed t test. Groups were considered to be significantly different for p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***).

Cloning and Expression of His<sub>6</sub>-smt3-TEV-mCGI-58_V10-K43—The coding sequence of mouse CGI-58 (mCGI-58) was available in a pSmu vector as described (5). An N-terminally truncated mCGI-58 variant, starting with the nucleotides cod ing for Val<sup>10</sup>, was subcloned into a modified pSmu vector carrying a tobacco etch virus (TEV) protease cleavage site. The oligonucleotides 5'-GTAACCTTGGATCCGTGGACTCGG-CAGACG-3' and 5'-GGAAACCTCCTGACATCAGTCTAC-TGTGTGGC-3' were used as forward and reverse primers, respectively. Then a stop codon was inserted by site-directed mutagenesis to truncate mCGI-58 after Lys<sup>43</sup> and produce the His<sub>6</sub>-smt3-TEV-mCGI-58_V10-K43 construct. This vector was transformed into BL21(DE3) Escherichia coli cells and cultures were grown in Luria broth (Miller, EMD Millipore Corp., Billerica, MA) medium containing 40 mg/liter of kanamycin up to an A<sub>600</sub> of 1.0 before induction with 0.5 mM isopropyl β-D-thiogalactopyranoside. After 3 h expression at 37 °C the cells were harvested by centrifugation for 20 min at 4 °C and 3,500 × g. 15N- and 13C-labeled His<sub>6</sub>-smt3-TEV-mCGI-58_V10-K43 was expressed in minimal medium containing 1 g/liter of [15N]NH₄Cl and 2 g/liter of [13C]glucose.

Purification of His<sub>6</sub>-smt3-TEV-mCGI-58_V10-K43—The cell pellet from a 1-liter culture was resuspended in 50 ml of buffer 1 (20 mM Tris-HCl, pH 7.8, 500 mM NaCl, 30 mM imidazole, 1% Nonidet P-40, 3.5 mM β-mercaptoethanol), 1 tablet of Roche EDTA-free protease inhibitor, 1 mg/ml of lysozyme, and 750 units of benzonase<sup>®</sup> nuclease HC (purity >90%, Novagen)). The cells were lysed by sonication and the soluble fraction was separated by centrifugation at 39,000 × g for 40 min at 4 °C. This fraction was incubated with 4 ml of Ni-NTA beads (Qiagen), pre-equilibrated in buffer 2 (20 mM Tris-HCl, pH 8.0, 350 mM NaCl, 10 mM imidazole, 3.5 mM β-mercaptoethanol), for 60–90 min at 4 °C on a nutator. The Ni-NTA beads were washed with 50 ml of buffer 2, buffer 3 (20 mM Tris-HCl, pH 8.0, 1000 mM NaCl, 10 mM imidazole, 3.5 mM β-mercaptoethanol), and buffer 4 (20 mM Tris-HCl, pH 8.0, 350 mM NaCl, 40 mM imidazole, 3.5 mM β-mercaptoethanol), respectively, to remove nonspecifically bound proteins. His<sub>6</sub>-smt3-TEV-mCGI-58_V10-K43 was eluted with 30 ml of buffer 5 (20 mM Tris-HCl, pH 8.0, 350 mM NaCl, 250 mM imidazole, 3.5 mM β-mercaptoethanol) and concentrated, and exchanged to buffer 7 (17.6 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.4 mM Na<sub>2</sub>PO<sub>4</sub>, 50 mM NaCl, 1 mM EDTA, 5 mM DTT, pH 6.0). For NMR experiments, 5% D<sub>2</sub>O were added to a 1 mM sample of peptide V10-K43 (within this text, this peptide is referred to as peptide “V10-K43”).

Preparation of the Synthetic Peptide “G18-E39”—A 22-residue peptide containing the amino acid sequence GSGWLTG-GLPTWCPTSTSHLKE corresponding to residues Glu<sup>18</sup>-Glu<sup>39</sup> of mCGI-58 (referred to as peptide “G18-E39”) was purchased from a commercial supplier (Peptide Special Laboratories, Heidelberg). For NMR experiments, it was dissolved in buffer 7 containing 100 mM DPC-<i>d</i><sub>16</sub> at a concentration of 1 mM and measured upon addition of 5% D<sub>2</sub>O.

NMR Experiments with Peptides V10-K43 and G18-E39—All experiments on peptides V10-K43 and G18-E39 were recorded in the presence of DPC micelles. Due to the low solubility of the peptides in aqueous solvents, assignment of CGI-58 peptides V10-K43 and G18-E39 in the absence of detergent was not feasible.

Standard backbone experiments (30) (HNCA, HN(CA)CO, HN(CA)Cα) of the peptide V10-K43 were recorded on a 600 MHz Bruker spectrometer equipped with an Avance I console and a cryogenically cooled TCI 5-mm probe. The side chain experiments HCCH-TOCSY and H(C(C)NH)-TOCSY were recorded on a 500 MHz Varian spectrometer equipped with a Unity Inova console and an HCN cryoprobe. An H(C(C)-CO)NH-TOCSY was recorded on a 750 MHz Bruker spectrometer equipped with a cryogenically cooled TCI 5-mm probe and an Avance III console.

15N- and 13C-dispersed three-dimensional NOESY experiments were recorded on a 700 MHz Varian spectrometer equipped with an Agilent dd2 console and an HCN salt-tolerant cryoprobe (150 ms mixing time) and a 900 MHz Bruker spectrometer with Avance II console and TCI cryoprobe (80 ms mixing time), respectively. All backbone and side chain experiments, with the exception of the (H)(C(C)-CO)NH-TOCSY, were recorded using non-uniform sampling where 15–20% of the indirect dimension grid was sampled using Poisson Gap Sampling (31). Heteronuclear three-dimensional NMR experiments were recorded at 310 K to minimize transversal relaxation times on residues immersed in the micelles. Concomitantly, this temperature reduced the dynamic range of the sample, as residues exposed to the solvent exchange more rapidly with water at elevated temperatures and therefore lost some of their otherwise high signal intensity. Homonuclear two-dimensional TOCSY and two-dimensional NOESY spectra of peptide G18-E39 were recorded on a 900 MHz Avance I Bruker spectrometer equipped with a cryogenically cooled probe at 305 K using 90 and 200 ms mixing times, respectively. Uniformly collected NMR spectra were processed with NMRpipe (32). Non-uniformly sampled spectra were processed with hmsISt in combination with NMRpipe (31, 33). All NMR spectra were visualized and analyzed with CcpNmr (34).

Relaxation experiments were recorded on the peptide V10-K43 at 310 K. Longitudinal (<i>T</i><sub>1</sub>) relaxation times were measured on a 600 MHz Avance I Bruker spectrometer equipped with a cryogenically cooled probe as a pseudo three-dimensional data set. Relaxation delays for <i>T</i><sub>1</sub> were 10, 50, 100, 150, 200, 300, 500, 800, 1000, 1200, 1500, and 1800 ms, respectively. Spin-spin (<i>T</i><sub>2</sub>)
relaxation times and heteronuclear NOE (15N[1H] NOE) experiments were recorded on an 800 MHz Bruker spectrometer equipped with an Avance III console and a room temperature probe. The relaxation delays used in the $T_2$ data series were 20, 40, 60, 80, 100, 120, 140, and 160 ms, respectively. The interleaved 15N[1H] NOE experiment was recorded with a 2-s saturation delay. Peak heights were integrated with CcpNmr and $T_1$ and $T_2$ times were also calculated within the CcpNmr software suit. 15N[1H] NOE peak heights were evaluated with relax (35, 36). 15N and 13C paramagnetic relaxation enhancements of V10-K43 were obtained from $T_1$ delay modulated 1H-15N HSQC and 1H-13C HSQC spectra, respectively, at 303 K. In both the above mentioned series of experiments, samples of V10-K43 were titrated with 0, 2, 4, 6, 8, and 10 mM gadolinium-diethylenetriamine pentaacetic acid-bismethylamide (Gd(DTPA-BMA)) and relaxation delays were 70, 150, 250, 350, 500, 750, 1000, 2000, 3000, and 5000 ms, respectively. Using the program relax, peak intensities were fitted as described (37) to obtain $T_1$ relaxation times. The spectra were recorded on an 800 MHz Bruker spectrometer equipped with an Avance III console and a TCI 5-mm cryogenically cooled probe.

Paramagnetic relaxation enhancement (PRE) values were extracted and converted to distance restraints according to published protocols (37, 38). We calculated the hydrodynamic radius of the DPC micelles to be ~30 Å ($r = 30$ Å) based on the translational diffusion coefficient measured by dynamic light scattering (described below). The constants $g$ (7.98 Å) and $k$ (253 mm$^{-1}$ Å$^3$) were used according to the literature (38). Gd(DTPA-BMA) was purchased as Gadodiamide from (Toronto Research Chemicals, Toronto, Canada) and added from a 60 mM stock in H$_2$O. PRE-derived distance restraints were weighted at 30% with respect to NOEs, upper and lower boundaries of ±2 Å were used.

NOEs from 15N- and 13C-dispersed NOEY-HSQC of V10-K43 and a homonuclear NOEY of peptide G18-E39 were picked, assigned, integrated, and converted to distance restraints in CcpNmr (34). Restraints for torsion angles were prepared with TALOS+ (39) and PRE-derived distance restraints were calculated as described above. 100 structures were calculated with a simulated annealing protocol using CYANA (40) and the 20 structures with the lowest energy target functions were chosen for deposition. Structures were visualized with PyMOL (41) and the quality of the structures was assessed with PSVS (42) and iCING (43). Circular Dichroism (CD) Spectroscopy—For CD spectroscopy, a sample of peptide V10-K43 was prepared at 0.76 mg/ml in buffer 7 and DPC as described above for the preparation of NMR samples. A corresponding baseline sample was prepared without peptide. Data were measured with a Jasco J-715 spectropolarimeter at 0.01-cm path length between 190 and 260 nm with 0.1-nm steps, 1-nm bandwidth, and 1-s averaging time at 50 nm min$^{-1}$ scanning speed. 10 spectra were recorded, averaged, and baseline corrected.

Dynamic Light Scattering—The hydrodynamic radius of micelles in the presence of peptide was measured to be 30 Å with dynamic light scatting (Protein Solutions DynaPro MS/X instrument, Protein Solutions Inc., Lakewood, NJ). The dynamic light scattering micelles with the peptides were measured at 5-s acquisition time, 30% laser power, and 20 acquisitions. As a reference, 100 mM DPC was measured in H$_2$O. The measured radius of 21 Å for the free micelle is in good agreement with the literature (38, 44, 45). The difference in micelle size is presumably due to higher salt and DPC concentration, which is a direct result of the peptide preparation process using spin concentrators.

Protein Data Bank (PDB) and Biological Magnetic Resonance Bank (BMRB) Accession Numbers—Coordinates and NMR resonance assignments have been deposited in the Protein Data Bank (PDB code 5A4H) (46) and Biological Magnetic Resonance Data Bank (BMRB code 25684) (47).

Results

The LD-binding Motif of CGI-58 Tolerates the Loss of Any Single Tryptophan Residue, but Not the Simultaneous Loss of Trp$^{21}$ and Trp$^{29}$ — Full-length mammalian CGI-58 localizes to LDs in differentiated 3T3-L1 adipocytes and COS-7 cells (Fig. 1A). This interaction involves the tryptophan-rich N terminus of CGI-58 (Trp$^{21}$, Trp$^{25}$, and Trp$^{29}$) in LD binding (15, 20, 49). CGI-58 lacking the first 31 residues or harboring changes in the three N-terminal Trp residues failed to co-localize to LDs (15), whereas conversion of Trp$^{21}$ to alanine (W21A) alone did not prevent the localization of CGI-58 to LDs or the activation of ATGL (Fig. 1C). Similarly, CGI-58 variants W25A and W29A retained their ability to localize to LDs; although somewhat reduced ATGL stimulation was observed for the variants W21A and W25A (Fig. 1, B and C).

Next, we generated variants with double amino acid exchanges, W21A/W25A, and W21A/W29A. Although the W21A/W25A variant localized to LDs and concomitantly activated ATGL with undiminished capacity the W21A/W29A variant failed to localize to LDs and to activate ATGL (Fig. 1, B and D). This strengthens the functional relevance of proper CGI-58 localization observed previously (15) and supports the proposed prominent role for the N-terminal region of CGI-58.

To investigate whether the N-terminal region self-sufficiently localizes to LDs, we expressed a YFP-tagged peptide ranging from Ser$^{19}$ to Ser$^{25}$ (CGI_19–35) in COS-7 cells and monitored its intracellular localization. The peptide localized to LDs in a manner reminiscent of wild type CGI-58 (Fig. 1, A and E). However, when we tested the peptide CGI_19–35 for its ability to activate the triacylglycerol (TG) hydrolase activity of ATGL, we did not observe any stimulating effect (Fig. 1F).

Resonance Assignments of the CGI-58 Peptides G18-E39 and V10-K43—To characterize the three-dimensional structure of the N-terminal LD binding region, we determined the solution structure using NMR spectroscopy. 96% of backbone and 74% of side chain resonances of the peptide V10-K43 bound to DPC micelles were assigned (Fig. 2A). The heteronuclear 15N- and 13C-dispersed NOEY-HSQC spectra of the peptide V10-K43 did not contain a sufficient number of cross-peaks for structure calculation, which is likely attributed to dynamics of the sample at 310 K. Therefore, we recorded homonuclear TOCSY and NOEY experiments on the synthetic 22-residue peptide mCGI-58_G18-E39 (G18-E39) at a lower temperature of 303 K. The resonance assignments from the longer peptide V10-K43 could be transferred and consequently, we assigned all NH and
Hα resonances of the unlabeled 22-residue peptide G18-E39 with the exceptions of the His³⁶-Hα proton and resonances corresponding to Gly¹⁸ at the N terminus. 82% of non-water exchangeable side chain protons were also assigned. Trp²¹ and Trp²⁵ Hε₁,Hδ₁ and Nε₁,Hζ₂ resonances were assigned with heteronuclear ¹H-¹⁵N HSQC and ¹⁵N-dispersed NOESY-HSQC spectra.

FIGURE 1. The N-terminal region of CGI-58 is a fully functional LD anchor. Substitution of its two terminal tryptophans abrogates the ability of CGI-58 to localize to LDs and to activate ATGL. A, confocal laser-scanning microscopy image of YFP tagged wild type (WT) CGI-58 expressed in oleate laden COS-7 cells (first column) co-localizes with LDs. HCS LipidTOX-stained LDs (second column) are overlaid with YFP-CGI-58 in third column. The fourth column represents the transmission image of the respective cells. Scale bars: 10 μm. B, testing the ability of CGI-58 and several CGI-58 variants to activate ATGL. All single Trp variants (W21A, W25A, and W29A) activate ATGL. The double variant W21A/W25A retains the full ability to activate ATGL, but W21A/W29A cannot activate ATGL. Presented data are one representative from three independent experiments, mean ± S.D. C, single Trp variants of CGI-58 co-localize with LDs. D, the double variant W21A/W25A co-localizes with LDs, but the double variant W21A/W29A does not. E, YFP-tagged CGI-58 peptide ranging from Ser¹⁹ to Ser³⁵ (CGI_19_–35) co-localizes to LDs. F, CGI_19_–35 does not activate ATGL.

FIGURE 2. Assignment of the peptides V10-K43 and G18-E39. A, ¹H-¹⁵N HSQC spectrum of the V10-K43 peptide bound to DPC micelles, with backbone NH and tryptophan side chain NH assignments. B, aromatic region of the homonuclear NOESY spectrum of the G18-E39 peptide, also bound to DPC micelles.
The Trp<sup>29</sup> He-1 resonance and additional Trp aromatic side chain resonances were assigned from the homonuclear spectra (Fig. 2B). Assignments were deposited in the BMRB accession number 25684.

The N-terminal Peptide of CGI-58 Reveals a Mostly Unstructured Anchor—The observed chemical shifts of a protein or peptide are sensitive indicators of α-helix and β-sheet elements when compared with average random coil shifts. Thus, the assignments of the N-terminal peptides of CGI-58 reveal initial per-residue information on secondary structure elements. In particular, downfield shifts of <sup>13</sup>Cα and <sup>13</sup>CO and upfield shifts of <sup>1</sup>Hα resonances with averaged changes of 2.6, 1.7, and 0.38 ppm, respectively, would indicate an α-helix. Upfield shifts with averaged changes of 1.4 ppm for <sup>13</sup>Cα and <sup>13</sup>CO, and downfield shifts of 0.38 ppm for <sup>1</sup>Hα, would indicate β-sheets (50–52). Examination of <sup>13</sup>Cα, <sup>1</sup>Hα, and <sup>13</sup>CO shifts of the CGI-58 peptide V10-K43 in DPC micelles did not provide an indication of α-helix or β-sheet elements, although a propensity for helix formation might be inferred for the region Gly<sup>20</sup>-Gly<sup>24</sup> (Fig. 3, A–C). Additionally, a circular dichroism (CD) spectrum of the peptide V10-K43 showed characteristics of a mostly unfolded peptide with a minimum around 200 nm (Fig. 3D). These experimental results are in good agreement with structure predictions. Secondary structure predictions indicate peptide V10-K43 to be partially unstructured, with a propensity to form α-helices (PSIPRED, Jpred (53, 54)). A homology model of CGI-58 based on the structure of the Aspergillus niger epoxide hydrolase also indicates helical and unstructured parts in the N terminus of CGI-58 (15).

Relaxation Analysis of the CGI-58 Peptide V10-K43 Confirms Binding to DPC Micelles and Independent Motion of Three Different Segments within the Peptide—To investigate the dynamic behavior of the individual residues of the CGI-58 peptide V10-K43, we recorded longitudinal (spin-lattice, <i>T</i><sub>1</sub>) and transversal (spin-spin, <i>T</i><sub>2</sub>) relaxation experiments and a set of heteronuclear NOE (<sup>15</sup>N[<sup>1</sup>H] NOE) experiments. <i>T</i><sub>1</sub> times increase when the tumbling rate of a residue slows down, whereas a decrease in the spin-spin relaxation (<i>T</i><sub>2</sub>) time corresponds to a decrease of tumbling rate and increase in protein size. The <sup>15</sup>N[<sup>1</sup>H] NOE experiment measures the change in steady-state populations of <sup>15</sup>N spins when the attached proton spins are saturated. This experiment is specifically sensitive to changes of the correlation time for internal motion and reflects variations in protein backbone dynamics on the pico- to nanosecond time scale. Reduced and negative NOEs correspond to rapid internal motion, whereas ratios close to 0.8 indicate more stable segments of proteins and peptides (55).

The relaxation experiments on the CGI-58 peptide V10-K43 revealed a clear separation of three distinct regions, namely flexible N- and C-terminal regions and a more rigid central region (Fig. 4). <i>T</i><sub>1</sub> times increased markedly between Trp<sup>21</sup> and Ser<sup>35</sup> (Fig. 4A), indicating reduced mobility. This is in agreement with the <i>T</i><sub>2</sub> values (Fig. 4B), which were less than 100 ms for residues between Trp<sup>21</sup> and Ser<sup>35</sup>. <i>T</i><sub>2</sub> times less than 100 ms in the LD binding region correspond to motion dynamics of a large (>20 kDa) protein. This indicates that this region comprises the LD anchor and is embedded in the LD mimicking micelle. Moreover, the rapid dynamics observed for the terminal regions indicate that these regions move independently of the LD anchor. This is further substantiated with the <sup>15</sup>N[<sup>1</sup>H] NOE experiment. Residues experiencing rapid internal motion flank a considerably more rigid core between Gly<sup>20</sup> and Thr<sup>32</sup> (Fig. 4C).

Paramagnetic Relaxation Enhancements Reveal the Immersion Depth of the Peptide Anchor—To elucidate the orientation of the CGI-58 peptide V10-K43 in DPC micelles and to determine the boundaries of the LD-binding motif, we recorded longitudinal relaxation experiments in the presence of various Gd(DTPA-BMA) concentrations. PREs collected in the presence of this highly water soluble and inert compound correlate with the insertion depths of peptide residues and can be con-
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experiments (Fig. 4). The central region ranging from Gly16 to dynamics in this region, as evidenced by the NMR relaxation times (\(T_1\)). All protons calculated to be within 28 Å of the hydrodynamic radius of the DPC micelles was calculated to be 30 Å in the presence of the CGI-58 peptide. For protons more than 28 Å the CGI-58 peptide. For protons more than 28 Å to distance restraints during structure calculation (37, 38). Based on dynamic light scattering experiments, the hydrodynamic radius of the DPC micelles was calculated to be 30 Å in the presence of the CGI-58 peptide. For protons more than 28 Å from the micelle center, only lower distance restraints were generated. All protons calculated to be within 28 Å of the micelle center correspond to residues ranging from Trp21 to Trp29 and thus reaffirm the depth of immersion of the LD anchor motif (Fig. 1). The paramagnetic restraints are deposited with the accession number 5a4h at the Protein Data Bank.

Solution Structure of the N-terminal LD Anchor of CGI-58 — To assess the differences in motion dynamics and establish the mode of binding of the peptide V10-K43, we calculated its solution structure immersed in DPC micelles. 533 NOE distance restraints were used for structure calculation, along with 36 backbone angular restraints (\(\phi\) and \(\psi\)) and 66 PRE-derived distance restraints. The complete summary of quality statistics and experimental restraints is provided in Table 2. As expected, the backbone dihedral angles \(\phi\) and \(\psi\) predominantly occupy coil regions of the Ramachandran plot (56). The flexible N- and C-terminal regions corresponding to residues Val10-Ala15 and Ser33-Lys43, respectively, are poorly defined due to the inherent dynamics in this region, as evidenced by the NMR relaxation experiments (Fig. 4). The central region ranging from Gly16 to Thr32 accounted for almost two-thirds (329 NOEs) of the NOE restraints and converged during structure calculation, revealing key aspects of LD binding. The predominantly hydrophobic and aromatic residues Ser19, Cys30 are immersed in the LD mimicking DPC micelles and constitute the LD anchor (Fig. 5). The left arm of this anchor comprises tryptophans 21 and 25 along with the leucines 22 and 26. These hydrophobic residues form a compact core along with a short helix between Gly20 and Gly24 (Fig. 5, B and C). A network of NOEs between tryptophan NHe-1 and NH\(_{\text{H}}\)-1 protons and the Leu\(_{\beta}\)-protons exemplifies these interactions (Fig. 6). Pro27 isolates Trp29 from the other tryptophans and together with Pro31 prevents the formation of a longer and more stable helix. The residue pairs Gly18/Ser19 and Cys30/Pro31 mark the interface between the DPC micelles and the solvent (Fig. 5C). A representation of the electrostatic potential on the solvent accessible surface of the peptide reveals the highly polar nature of the terminal segments and a predominantly hydrophobic LD anchor (Fig. 5, B and D).

Discussion

In this study, we describe the three-dimensional solution structure of the N-terminal LD anchor of CGI-58. Three anchor points (Trp21, Trp25, and Trp29) act synergistically to tether CGI-58 stably to LDs. The peptide sequence immersed
within the LD comprises amino acids Ser\textsuperscript{19}-Cys\textsuperscript{30}. We demonstrate that a slightly longer CGI-58 sequence stretching from Ser\textsuperscript{19}-Ser\textsuperscript{35} also recruits the otherwise cytosolic yellow fluorescent protein to LDs. However, this LD anchor lacks the ability to activate ATGL, indicating that other regions of CGI-58 are necessary for ATGL activation. The data presented here also corroborate earlier studies that LD binding of CGI-58 is a strict requirement for ATGL activation (15).

Single amino acid mutagenesis of any of the three tryptophans of the CGI-58 LD anchor and a variant lacking two tryptophan side chains (W\textsubscript{21A}/W\textsubscript{25A}) had no effect on CGI-58 LD co-localization. The ability of the CGI-58 variants to activate ATGL was reduced at most by one-third. In contrast, when we tested a W\textsubscript{21A}/W\textsubscript{29A} variant, the ability of CGI-58 to localize to LDs and to activate ATGL was completely abrogated. The unstructured nature of the LD anchor enables conformational flexibility and functional promiscuity. Therefore, it is conceivable that the CGI-58 LD anchor undergoes a conformational change upon CGI-58 binding to ATGL. The LD anchor might be necessary for the correct orientation of CGI-58 on LDs, which provides the platform for interaction with ATGL, or for positioning TGs favorably with respect to CGI-58 bound ATGL. Alternatively, CGI-58 might serve as mediator to transfer released FFAs from LD-bound ATGL to the water-soluble and cytosolic fatty acid-binding proteins, yet the LD anchor motif of CGI-58 is not required for binding to fatty acid-binding proteins or ATGL (15, 16). Again, the correct positioning of CGI-58 with respect to all interaction partners could be realized via the LD-anchor. Obviously, additional structural data of the involved binary and ternary complexes are required to learn more about this complex network.
The structures of membrane proteins have become a prominent and rapidly expanding field of structural biology, due to novel experimental and methodological breakthroughs. Structural studies of proteins acting at the water-lipid or membrane interface pose a tremendous experimental challenge. Consequently, the interaction of proteins with LDs remains largely uncharted territory. CGI-58 was initially demonstrated to bind to LDs and perilipin 1 simultaneously in adipocytes. Upon activation of lipolysis, CGI-58 dissociates from perilipin 1 and forms a LD-bound complex with ATGL (57). Perilipin 1 independent binding of CGI-58 to LDs was demonstrated in COS-7 cells (15, 58). Cell types that do not express perilipin 1 often express other members of the perilipin family (perilipins 2–5). Perilipin 5 interacts with CGI-58 and ATGL in a mutually exclusive manner (59) and the interactions of CGI-58 with perilipins 2 and 3 appear to be functionally less significant (60).

Only a few structures of proteins that interact with LDs have been solved. Previously, Dunne and colleagues (61) showed the NMR structure of two CTP:phosphocholine cytidylyltransferase peptides in atomic detail on two overlapping 33- and 22-residue peptides, which span most of the amphipathic predominantly \( \alpha \)-helical membrane-binding domain of rat CTP: phosphocholine cytidylyltransferase. The structural work on these peptides was performed in a membrane-binding context, yet Drosophila orthologues of CTP:phosphocholine cytidylyltransferase have been shown to localize to LDs as well (62, 63).

Additionally, structures of a few soluble orthologues or domains of LD-binding proteins have been characterized (64, 65). The soluble C-terminal domain of the patatin family member TIP47 (perilipin 3) was solved nearly a decade ago (66), but the structure of the LD binding domain remains elusive. Intriguingly, the crystal structure of human monoacylglycerol lipase has been determined (67–69) and recently Nasr and colleagues (70) demonstrated that the cap region of human monoacylglycerol lipase interacts with nanodisc phospholipid micelles as LD mimics. The utilization of two independent LD binder poses the mechanism by which it binds to LDs using DPC micelles as LD mimics. The utilization of two independent LD binding arms reveals an intriguing strategy to protect CGI-58 function, because the right anchor arm comprising Trp29 abrogates the function of CGI-58, because both anchor arms are affected by the substitutions. The presence of two independently acting arms of the LD anchor could prevent defective anchoring as a result of malfunction in one LD anchor arm.

Selective inhibition of LD anchoring by CGI-58, or CGI-58 interactions with fatty acid-binding proteins, ATGL or perilipins, presents an opportunity for selective therapeutic targeting of lipid metabolism and peroxisome proliferator-activated receptor regulated gene expression. An essentially identical sequence to the LD anchor motif of CGI-58 can be found at the N terminus of a close relative of CGI-58, \( \alpha/\beta \) hydrolase 4 (ABHD4) (55% sequence identity). Unlike CGI-58, ABHD4 is an active serine hydrolase and has been shown to hydrolyze N-acyl phosphatidylethanolamines (NAPEs) and lyso-N-acyl phosphatidylethanolamines (73). Moreover, ABHD4 was recently demonstrated to be a regulator of multiple classes of N-acyl phospholipids in the mammalian nervous system (74). From a drug design perspective the highly similar LD anchor of CGI-58 and ABHD4 can be both an opportunity and a limitation. Molecules that hinder CGI-58 LD anchoring may potentially affect ABHD4 activity as well. This would limit the specificity of an approach to target the LD anchor of CGI-58, but might also present a chance to trigger synergistic effects by influencing the activities of both proteins.

In summary, we show the structure of a LD anchor and describe the mechanism by which it binds to LDs using DPC micelles as LD mimics. The utilization of two independent LD binding arms reveals an intriguing strategy to protect CGI-58 against the loss of LD binding activity and highlights the importance of proper CGI-58 binding to LDs.

**Author Contributions—** A.B., H. M. N., H. A., G. W., K. Z., and M. O. conceived and designed the experiments. A. B., H. M. N., H. A., C. J. P., R. E. L., K. Z., and M. O. analyzed the data. A. B., H. M. N., H. A., G. W., K. Z., and M. O. wrote the paper. All authors approved the final version of the manuscript.

**Acknowledgments—** We acknowledge the assistance of Astrid Gruber, Petra Ebner, and Pia Gruber at early stages of the project and we are grateful to Jim Zhen-Yu Sun and Barbara Ogórek for helpful discussions. Also, we thank Luna Scientific Storytelling, LLC for invaluable input and advice during the preparation of this manuscript. The IMB Graz Microscopy and Imaging Resource, BioTechMed, NAWI Graz is gratefully acknowledged for access to their facilities. Maintenance and operation of NMR instruments was supported in part by the National Institutes of Health Grant P41 EB002026. The authors gratefully acknowledge support from NAWI Graz.
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References

1. Guo, Y., Cordes, K. R., Farese, R. V., Jr., and Walther, T. C. (2009) Lipid droplets at a glance. J. Cell Sci. 122, 749–752

2. Lass, A., Zimmermann, R., Haemmerle, G., Riederer, M., Schoiswohl, G., Schweiger, M., Kenesberger, P., Strauss, J. G., Gorkiewicz, G., and Zechner, R. (2006) Adipose triglyceride lipase-mediated lipolysis of cellular fat stores is activated by CGI-58 and defective in Chanarin-Dorfman Syndrome. Cell Metab. 3, 309–319

3. Oberer, M., Boeszoermenyi, A., Nagy, H. M., and Zechner, R. (2011) Recent insights into the structure and function of comparative gene identification-58. Curr. Opin. Lipidol. 22, 149–158

4. Lass, A., Zimmermann, R., Oberer, M., and Zechner, R. (2011) Lipolysis: a highly regulated multi-enzyme complex mediates the catabolism of cellular fat stores. Prog. Lipid Res. 50, 14–27

5. Fischer, J., Lefèvre, C., Morava, E., Mussini, J. M., Lafortêt, P., Negre-Salvayre, A., Lathrop, M., and Salvayre, R. (2007) The gene encoding adipose triglyceride lipase (PNPLA2) is mutated in neutral lipid storage disease with myopathy. Nat. Genet. 39, 28–30

6. Radner, P. F., and Fischer, J. (2014) The important role of epidermal triacylglycerol metabolism for maintenance of the skin permeability barrier function. Biochim. Biophys. Acta 1841, 409–415

7. Schweiger, M., Lass, A., Zimmermann, R., Eichmann, T. O., and Zechner, R. (2009) Neutral lipid storage disease: genetic disorders caused by mutations in adipose triglyceride lipase/PGIN2 or CGI-58/ABHD5. Annu. J. Physiol. Endocrinol. Metab. 297, E289–296

8. Zierler, K. A., Zechner, R., and Haemmerle, G. (2014) Comparative gene identification-58/α/β hydrolase domain 5: more than just an adipose triglyceride lipase activator? Curr. Opin. Lipidol. 25, 102–109

9. Braasemle, D. L., Levin, D. M., Adler-Wailes, D. C., and Londos, C. (2000) The lipolytic stimulation of 3T3-L1 adipocytes promotes the translocation of hormone-sensitive lipase to the surfaces of lipid storage droplets. Biochim. Biophys. Acta 1483, 251–262

10. Cerk, I. K., Salzburger, B., Boeszoermenyi, A., Heier, C., Pillip, C., Roamauch, M., Schweiger, M., Cornaciu, I., Lass, A., Zimmermann, R., Zechner, R., and Oberer, M. (2014) A peptide derived from G0/G1 switch gene-2 acts as noncompetitive inhibitor of adipose triglyceride lipase. J. Biol. Chem. 289, 32559–32570

11. Chung, C., Doll, J. A., Gattu, A. K., Shgruere, C., Cornwell, M., Fitchev, P., and Crawford, S. E. (2008) Anti-angiogenic pigment epithelium-derived factor regulates hepatocyte triglyceride content through adipose triglyceride lipase (ATGL). J. Hepatol. 48, 471–478

12. Cornaciu, I., Boeszoermenyi, A., Lindermuth, H., Nagy, H. M., Cerk, I. K., Ebner, C., Salzburger, B., Gruber, A., Schweiger, M., Zechner, R., Lass, A., Zimmermann, R., and Oberer, M. (2011) The minimal domain of adipose triglyceride lipase (ATGL) ranges until leucine 254 and can be activated and inhibited by CGI-58 and G0S2, respectively. Plos One 6, e26349

13. Egan, J. J., Greenberg, A. S., Chang, M. K., Wek, S. A., Moos, M. C. Jr., and Londos, C. (1992) Mechanism of hormone-stimulated lipolysis in adipocytes: translocation of hormone-sensitive lipase to the lipid storage droplet. Proc. Natl. Acad. Sci. U.S.A. 89, 8537–8541

14. Ellong, E. N., Soni, K. G., Bui, Q. T., Sougrat, R., Golinelli-Cohen, M. P., and Bonifacino, J. S. (2009) Coatomer-dependent protein delivery to lipid droplets. J. Cell Biol. 183, 1383–1394

15. Young, S. G., and Zechner, R. (2013) Biochemistry and pathophysiology of intravascular and intracellular lipolysis. Genes Dev. 27, 459–484

16. Ferragut, R. M., Moore, H. P., Krishnamoorthy, R., and Rathod, M. (2009) Perilipin controls lipolysis by regulating the interactions of AB-hydrolase containing 5 (Abhd5) and adipose triglyceride lipase (Atgl). J. Biol. Chem. 284, 34538–34544

17. Eichmann, T. O., Kumari, M., Haas, I. T., Farese, R. V., Jr., Zimmermann, R., Lass, A., and Zechner, R. (2012) Studies on the substrate and stereo/regioselectivity of adipose triglyceride lipase, hormone-sensitive lipase, and diacylglycerol-O-acyltransferases. J. Biol. Chem. 287, 41446–41457

18. Schweiger, M., Schoiswohl, G., Lass, A., Radner, F. P., Haemmerle, G., Malli, R., Graier, W., Cornaciu, I., Oberer, M., Salvayre, R., Fischer, J., Zechner, R., and Zimmermann, R. (2008) The C-terminal region of human adipose triglyceride lipase affects enzyme activity and lipid droplet binding. J. Biol. Chem. 283, 17211–17220

19. Zimmermann, R., Strauss, J. G., Haemmerle, G., Schoiswohl, G., Birner-Grunenberger, R., Riederer, M., Lass, A., Neuberger, G., Eisenhaber, F., Hermetter, A., and Zechner, R. (2004) Fat mobilization in adipose tissue is affected by adipose triglyceride lipase. Science 306, 1383–1386

20. Ferenta, A. E., and Wagner, G. (2000) NMR spectroscopy: a multifaceted approach to macromolecular structure. Q. Rev. Biophys. 33, 29–65

21. Hyberts, S. G., Milbradt, A. G., Wagner, A. B., Arthanari, H., and Wagner, G. (2000) NMR spectroscopy: a multidimensional approach to macromolecular structure. Q. Rev. Biophys. 33, 29–65

22. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J. Biomol. NMR 6, 273–293

23. Hyberts, S. G., Robson, S. A., and Wagner, G. (2013) Exploring signal-to-noise ratio and sensitivity in non-uniformly sampled multi-dimensional NMR spectra. J. Biomol. NMR 55, 167–178
72. Walther, T. C., and Farese, R. V., Jr. (2012) Lipid droplets and cellular lipid metabolism. *Annu. Rev. Biochem.* **81**, 687–714
73. Simon, G. M., and Cravatt, B. F. (2006) Endocannabinoid biosynthesis proceeding through glycerophospho-N-acyl ethanolamine and a role for α/β-hydrolase 4 in this pathway. *J. Biol. Chem.* **281**, 26465–26472
74. Lee, H. C., Simon, G. M., and Cravatt, B. F. (2015) ABHD4 regulates multiple classes of N-acyl phospholipids in the mammalian central nervous system. *Biochemistry* **54**, 2539–2549
75. Laskowski, R. A., Rullmann, J. A., MacArthur, M. W., Kaptein, R., and Thornton, J. M. (1996) AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *J. Biomol. NMR* **8**, 477–486