Adipose triglyceride lipase-mediated lipolysis of cellular fat stores is activated by CGI-58 and defective in Chanarin-Dorfman Syndrome

Achim Lass,[1,2] Robert Zimmermann,[1,2] Guenter Haemmerle,[1] Monika Riederer,[1] Gabriele Schoiswohl,[1] Martina Schweiger,[1] Petra Kienesberger,[1] Juliane G. Strauss,[1] Gregor Gorkiewicz,[1] and Rudolf Zechner[1,*]

[1] Institute of Molecular Biosciences, University of Graz, Heinrichstrasse 31, A-8010 Graz, Austria
[2] These authors contributed equally to this work.
*Correspondence: rudolf.zechner@uni-graz.at

Summary

Adipose triglyceride lipase (ATGL) was recently identified as an important triacylglycerol (TG) hydrolase promoting the catabolism of stored fat in adipose and nonadipose tissues. We now demonstrate that efficient ATGL enzyme activity requires activation by CGI-58. Mutations in the human *CGI-58* gene are associated with Chanarin-Dorfman Syndrome (CDS), a rare genetic disease where TG accumulates excessively in multiple tissues. CGI-58 interacts with ATGL, stimulating its TG hydrolase activity up to 20-fold. Alleles of *CGI-58* carrying point mutations associated with CDS fail to activate ATGL. Moreover, CGI-58/ ATGL coexpression attenuates lipid accumulation in COS-7 cells. Antisense RNA-mediated reduction of CGI-58 expression in 3T3-L1 adipocytes inhibits TG mobilization. Finally, expression of functional CGI-58 in CDS fibroblasts restores lipolysis and reverses the abnormal TG accumulation typical for CDS. These data establish an important biochemical function for CGI-58 in the lipolytic degradation of fat, implicating this lipolysis activator in the pathogenesis of CDS.

Introduction

The storage of cellular triacylglycerol (TG) represents a common strategy in eukarya to fuel constant energy production in a world of infrequent nutrient supply. In vertebra including humans, excess nutritional carbohydrates and fat are efficiently converted into TG and deposited in adipose tissue. Functional energy homeostasis depends on a finely tuned balance of TG storage and mobilization. Dysfunction of the multifactorial regulatory network that links adipose tissue, the digestive tract, and nervous system via hormones, cytokines, and adipokines (Flier, 2004), disrupts the equilibrium of lipid synthesis and degradation resulting in prevalent metabolic diseases, such as obesity and type 2 diabetes (Shulman, 2000; Stumvoll et al., 2005).

Catabolism of TG storage depots and mobilization of free fatty acids (FFA) in adipocytes and other cell types depend on lipases. Lipolytic activities in adipose tissue modulate the concentration of FFA in plasma, which, when chronically elevated, exert a lipotoxic effect on many nonadipose tissues (Schaffer, 2003; Unger, 2002) and contribute to the development of insulin resistance (Boden and Shulman, 2002). Current models propose that two lipases are involved in the hydrolysis of TG in adipose and nonadipose tissues. The classical enzyme, hormone-sensitive lipase (HSL), hydrolyses TG, diglycerides (DG), and monoglycerides (MG), with the highest specific activity against DG (Belfrage et al., 1978). β-adrenergic stimulation of adipocytes and the subsequent protein kinase A-dependent phosphorylation of HSL and perilipin trigger the translocation of HSL from the cytoplasm to the lipid droplet and induce neutral lipid hydrolysis (Egan et al., 1992). Yet, studies in HSL-deficient mice showed that the enzyme is rate limiting in vivo for the catabolism of DG but not for TG (Haemmerle et al., 2002). Thus, an upstream enzyme in the lipolytic pathway was predicted and identified, adipose tri-

glyceride lipase (ATGL) (Zechner et al., 2005; Zimmermann et al., 2004). ATGL specifically removes the first fatty acid from the TG molecule generating FFA and DG. The drastic impairment of fat cell lipolysis in ATGL inhibition studies in vitro (Zimmermann et al., 2004; Smirnova et al., 2006) as well as the nutritional and insulin dependent regulation of ATGL gene expression in adipose tissue of mice (Villena et al., 2004; Kershaw et al., 2006) implied a key function for this enzyme in the lipolytic degradation of stored fat. The importance of the ATGL orthologs in *Drosophila melanogaster* (*brummer*) (Gronke et al., 2005) and *Sacharomyces cerevisiae (TGL4)* (Kurat et al., 2006) in vivo was reported recently where deficiency of ATGL led to obese phenotypes.

Human genetic disorders that affect lipolysis due to mutations in the genes for HSL or ATGL have not been identified. However, a defect in the lipolytic catabolism of stored TG has been discussed in association with a rare autosomal recessive inborn error of neutral lipid metabolism—Chanarin-Dorfman Syndrome (CDS [MIM 27630]) (Chanarin et al., 1975; Dorfman et al., 1974). CDS is clinically characterized by ichthyosis, variably accompanied by liver steatosis, cardiomyopathy, ataxia, hearing loss, and mental retardation. Affected individuals accumulate neutral lipids in multiple tissues leading to the alternative designation "neutral lipid storage disease." Lipid accumulation does not result from increased cellular FFA uptake or TG synthesis rates (Williams et al., 1988). Instead, it was suggested that a defect in the catabolism of stored TG underlies the observed phenotypes (Williams et al., 1991). However, when the gene for comparative gene identification, 58 (CGI-58, identical to α/β-hydrolase domain-containing protein 5, ABHD5), was identified as causative for CDS (Lefèvre et al., 2001), its involvement in neutral lipid catabolism remained unexplained. CGI-58 belongs to the esterase/thioesterase/lipase subfamily of proteins structurally characterized by the presence of α/β-hydrolase folds. The
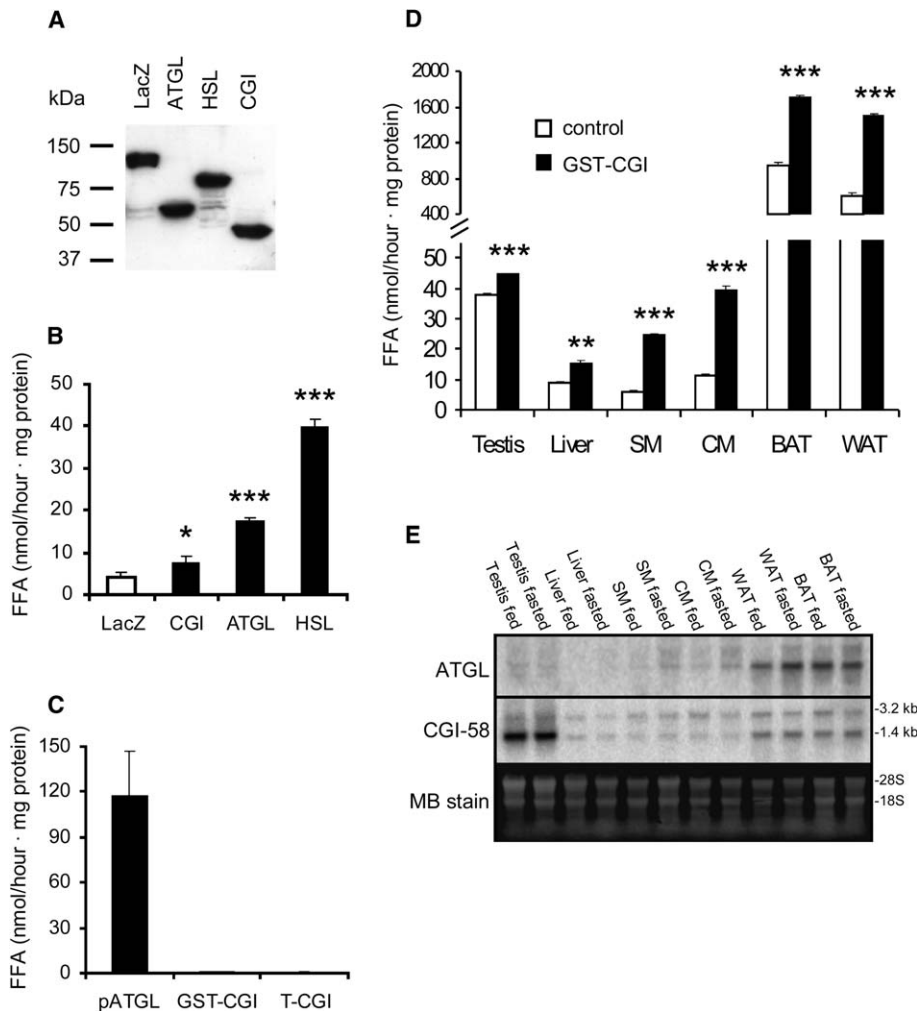
Figure 1. CGI-58 increases the TG hydrolase activity in cell and tissue extracts

A) Murine ATGL, HSL, and CGI-58 (CGI) were cloned into a His-tagged pcDNA4/HisMax expression vector, and recombinant proteins were expressed in COS-7 cells. β-galactosidase (LacZ) was used as a control. His-tagged proteins were detected with a monoclonal anti-His antibody in Western blotting experiments using cell extracts (20 μg protein).

B and C) (**B**) TG hydrolase activity of cell extracts of transfected cells or (**C**) of purified His-tagged ATGL (pATGL, 100 ng/assay), GST-tagged CGI-58 (GST-CGI, 1 μg/assay), and thrombin-treated purified CGI-58 (T-CGI, 1 μg/assay) was determined using a radiolabeled triolein substrate.

D) TG hydrolase activities in cytosolic extracts of mouse tissues were determined either in the presence or in the absence of purified GST-CGI (100 ng/assay).

E) Northern blotting analysis of CGI-58 and ATGL mRNA expression in various mouse tissues obtained from fed and fasted mice (SM, skeletal muscle; CM, cardiac muscle; WAT, white adipose tissue; BAT, brown adipose tissue). The amount of RNA loaded onto the blot was visualized using methylene blue (MB) staining.

Data for TG hydrolase activity assays are presented as mean ± SD and represent at least three independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001).

putative active serine within the canonical esterase/lipase motif GXSXG is replaced by asparagine in CGI-58 (Lefèvre et al., 2001). In adipose tissue, CGI-58 binds to intracellular lipid droplets by interaction with perilipin (Subramanian et al., 2004; Yamaguchi et al., 2004). The different gene defects presently known for CDS include truncations, deletions, and point mutations implying a loss of CGI-58 function. The present study is based on the premise that CGI-58 acts as a TG hydrolase or assists known TG hydrolases in the lipolytic breakdown of stored fat.

### CGI-58 is not a lipase but enhances lipolytic activity in tissue extracts

The effect of CGI-58 on cellular TG hydrolase activity was tested in simian virus-40-transformed monkey kidney cells (COS-7) transfected with cDNA clones expressing His-tagged murine CGI-58, ATGL, HSL, or β-galactosidase (LacZ). Western blotting confirmed the expression and the correct molecular mass of the respective proteins (Figure 1A). In TG hydrolase assays with cell extracts of transfected cells (Figure 1B), overexpression of CGI-58 increased the TG hydrolase activity by 1.8-fold compared to LacZ-containing extracts. In comparison, transfection of cells with ATGL and HSL had a more pronounced effect and increased TG hydrolase activities by 4- and 9-fold, respectively (Figure 1B).

Purified CGI-58 does not exhibit intrinsic TG hydrolase activity. A glutathione-S-transferase (GST) CGI-58 fusion protein was expressed in transformed *S. cerevisiae* and purified. In contrast to purified ATGL (pATGL), GST-tagged CGI-58 (GST-CGI) did not exhibit TG hydrolase activity even after thrombin-mediated removal of the GST tag (T-CGI) (Figure 1C). However, addition of GST-CGI to tissue lysates of testis, liver, skeletal muscle, cardiac muscle, and white or brown adipose tissue increased their TG hydrolase activity up to 4-fold (Figure 1D).

Northern blotting analysis revealed that CGI-58 is expressed in virtually all tissues analyzed (Figure 1E). Both CGI-58 mRNA species, which vary in their 3′ untranslated regions (Subramanian et al., 2004), are most abundant in testis followed by adipose tissue, liver, and muscle. In comparison, ATGL mRNA levels are highest in adipose tissue followed by testis, cardiac muscle, and skeletal muscle. Notably, the activation of lipolysis by exogenous CGI-58 inversely correlates with its tissue-specific mRNA levels, suggesting that in tissues of low expression, CGI-58 might be rate limiting for the lipolytic reaction.

CGI-58 specifically activates ATGL, evidence for protein-protein interaction

The effect of CGI-58 on the hydrolytic activity of known TG lipases was analyzed in mixtures of COS-7 cell extracts

A

B

C

D

E
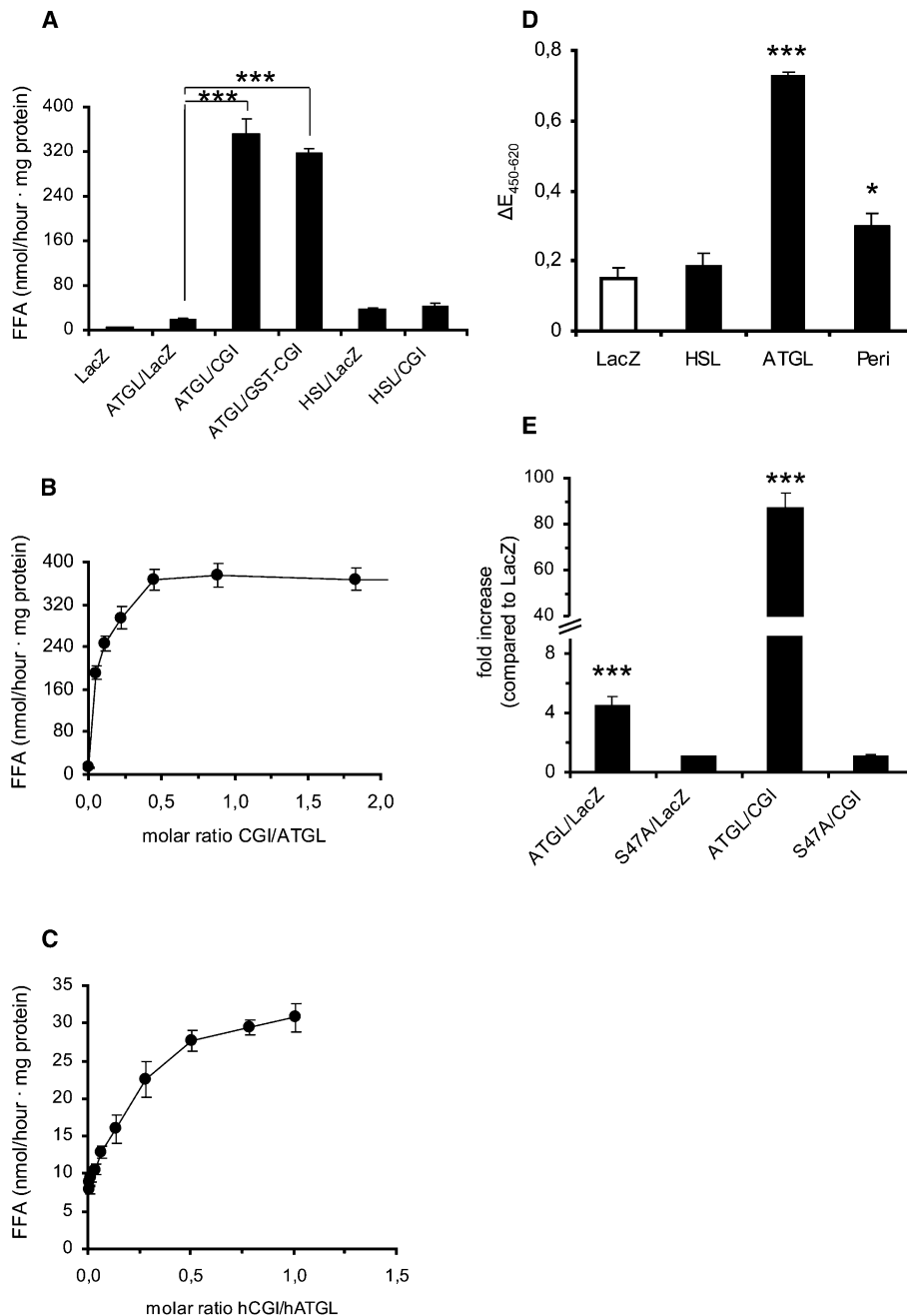
Figure 2. CGI-58 specifically activates TG hydrolase activity of ATGL

A) Cell extracts of COS-7 cells expressing ATGL or HSL were mixed with extracts containing CGI-58 (CGI), LacZ, or purified GST-CGI (100 ng/assay), as indicated, and TG hydrolase activity was determined. **B and C)** Dose-dependent effect of CGI-58 on ATGL TG hydrolase activity. Cell extracts containing (**B**) murine ATGL or (**C**) human ATGL were mixed with increasing amounts of extracts containing murine or human CGI-58 (hCGI), respectively, and were subjected to TG hydrolase activity assays. The amount of His-tagged ATGL and CGI-58 were determined by Western blotting analysis and densitometrical quantitation.

D) For protein-protein interaction studies, ELISA plates were coated with GST-CGI or GST and incubated with COS-7 cell extracts containing His-tagged LacZ, HSL, ATGL, or perilipin A (Peri). Binding of proteins was detected using anti-His primary- and horseradish peroxidase-conjugated secondary antibodies. The absorbance of the peroxidase reaction was determined photometrically using tetramethylbenzidine as substrate. The absorbance of GST-coated wells (negative control) was subtracted from that obtained with GST-CGI.

E) Cell extracts containing ATGL or the ATGL mutant S47A were mixed with extracts containing CGI-58 or LacZ, and TG hydrolase activity was determined. TG hydrolase activity is expressed as fold increase compared to the LacZ control.

All data are presented as mean ± SD and represent at least three independent experiments ($*p < 0.05$, $***p < 0.001$).

expressing murine ATGL or HSL (Figure 2A). The addition of CGI-58 to ATGL-containing extracts enhanced TG hydrolase activity by 20-fold compared to extracts containing ATGL alone. A comparable increase in ATGL activity was observed in the presence of purified GST-CGI. In contrast, CGI-58 had no effect on the enzymatic activity of extracts containing HSL, suggesting that the substantial activation of TG hydrolysis by CGI-58 was specific for ATGL. In dose-response experiments, maximal ATGL activity was achieved at a molar CGI-58/ATGL ratio of 0.5 to 1.0 (Figure 2B), indicating that the effect of CGI-58 on TG hydrolase activity is saturable and limited by the amount of ATGL in the assay system. Human CGI-58 (hCGI) and human ATGL (hATGL) revealed similar results. As shown in Figure 2C, hCGI increased the activity of hATGL in a dose-dependent and saturable

manner. In comparison to the mouse ortholog, the magnitude of the maximal effect on ATGL activation was smaller (4-fold versus 20-fold) suggesting a quantitative species-dependent difference in the biological activity of the human and mouse proteins.

Protein-protein interaction between murine CGI-58 and ATGL was demonstrated in ELISA experiments. Purified GST-CGI was bound to ELISA plates and incubated with cell extracts containing HSL or ATGL. His-tagged LacZ and perilipin A served as negative and positive controls, respectively (Subramanian et al., 2004; Yamaguchi et al., 2004). Compared to LacZ, an increased binding was observed for ATGL and, to a lower extent, for perilipin A, suggesting a specific interaction of these two proteins with CGI-58 (Figure 2D). In contrast, HSL did not bind to GST-CGI above the level of the LacZ control.
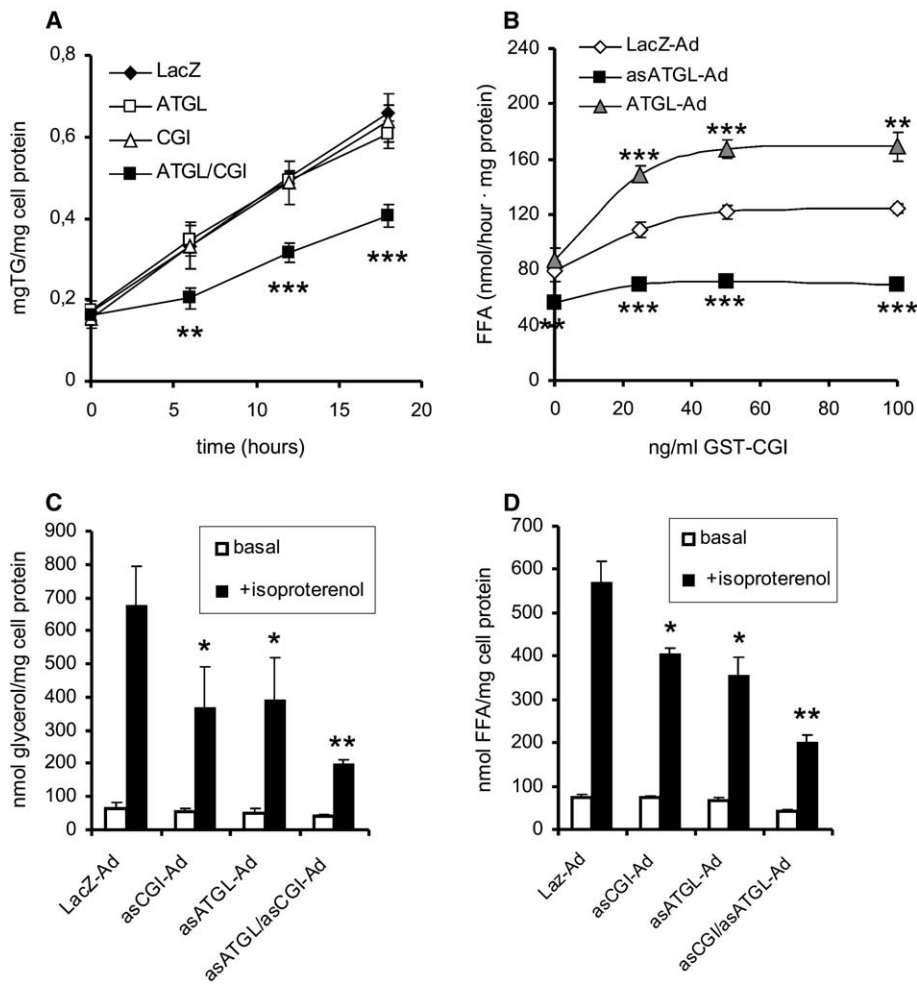
Figure 3. Effect of CGI-58 on cellular TG metabolism

A) COS-7 cells were transfected with LacZ, ATGL, CGI-58, or both ATGL and CGI-58. Thirty-six hours after transfection, cellular TG accumulation was promoted by the addition of oleic acid (0.5 mM). Cellular TG content was determined at the indicated time points.

B) Recombinant adenovirus encoding LacZ (LacZ-Ad), murine ATGL (ATGL-Ad), or murine antisense ATGL (asATGL-Ad) were used to infect 3T3-L1 adipocytes on day 5 of differentiation. Two days after infection, the CGI-58 activatable TG hydrolase activity of cytosolic lysates was determined by incubating lysates in the absence and presence of various amounts of purified GST-CGI using radiolabeled triolein as substrate.

C and D) Release of glycerol (**C**) and FFA (**D**) into the culture medium of 3T3-L1 adipocytes in the absence (basal) or presence of 10 μM isoproterenol. Cells were infected on day 5 of differentiation using adenovirus encoding LacZ-Ad, asCGI-58-Ad, asATGL-Ad, or both asATGL-Ad and asCGI-Ad. Two days after infection, adipocytes were incubated in DMEM containing 2% FFA-free BSA for 1 hr, and the release of glycerol and FFA was determined.

Data are presented as mean ± SD and represent three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

To demonstrate that CGI-58-mediated activation of TG hydrolysis is based on a functional TG hydrolase activity of ATGL, we transfected COS-7 cells either with murine wild-type ATGL or an ATGL construct carrying a single point mutation that expresses an enzyme with the putative active site serine at position 47 replaced by alanine (S47A). Compared to ATGL-containing cell extracts, S47A extracts exhibited no measurable TG hydrolase activity above the level of LacZ transfected cells (Figure 2E). When cell extracts containing S47A and CGI-58 were combined, no stimulation of TG hydrolysis was observed indicating that the CGI-58-mediated activation of TG hydrolase activity was dependent on enzymatically functional ATGL.

Modulation of CGI-58 and/or ATGL expression affects cellular lipolysis and TG storage

In COS-7 cells, endogenous expression of ATGL and CGI-58 is insufficient to be detected by Northern blotting analysis. Therefore, these cells were transfected with expression plasmids for the murine proteins to assess whether CGI-58 and ATGL, singly or in combination, affect TG accumulation in cells. In culture medium containing 0.5 mM oleate, the rate of TG accumulation was identical for cells expressing LacZ, ATGL, and CGI-58 alone (Figure 3A). In contrast, TG accumulation was drastically reduced in cells expressing both ATGL and CGI-58, consistent with the assumption that CGI-58-mediated activation of ATGL promotes TG lipolysis thereby lowering TG storage.

The effect of modulating the level of ATGL expression on the ability of CGI-58 to activate lipolysis was evaluated in adipocytes. Differentiated 3T3-L1 cells were infected with an adenovirus encoding murine ATGL (ATGL-Ad) or with an adenovirus encoding murine ATGL antisense RNA (asATGL-Ad) (Figure 3B). Without the addition of CGI-58, the TG hydrolase activity in the cytosolic cell extracts was similar in cells infected with LacZ and ATGL-Ad and reduced by 40% when cells were infected with asATGL-Ad. Upon the addition of increasing amounts of exogenous CGI-58 to the cell extracts, the highest stimulatory effect on TG hydrolase activity was obtained in ATGL-Ad infected cells. In contrast, when ATGL expression was silenced by asATGL-Ad, the CGI-58 inducible TG hydrolase activity was drastically reduced suggesting that ATGL represents the major target for CGI-58-mediated activation of lipolysis in adipocytes.

This conclusion was further supported when the effect of lowered CGI-58 and/or ATGL expression on glycerol and FFA release was studied in 3T3-L1 adipocytes. Infection of 3T3-L1 cells with asCGI-Ad and asATGL-Ad reduced glycerol release by 46% and 42%, respectively, when lipolysis was induced by isoproterenol (Figure 3C). Coinfection of cells with both asCGI-Ad and asATGL-Ad resulted in a 71% reduction compared to LacZ-transfected 3T3-L1 adipocytes. Similarly, the FFA release was reduced by 45%, 46%, and 72%, respectively (Figure 3D). These data demonstrate that ATGL activation by CGI-58 controls TG mobilization in adipocytes.
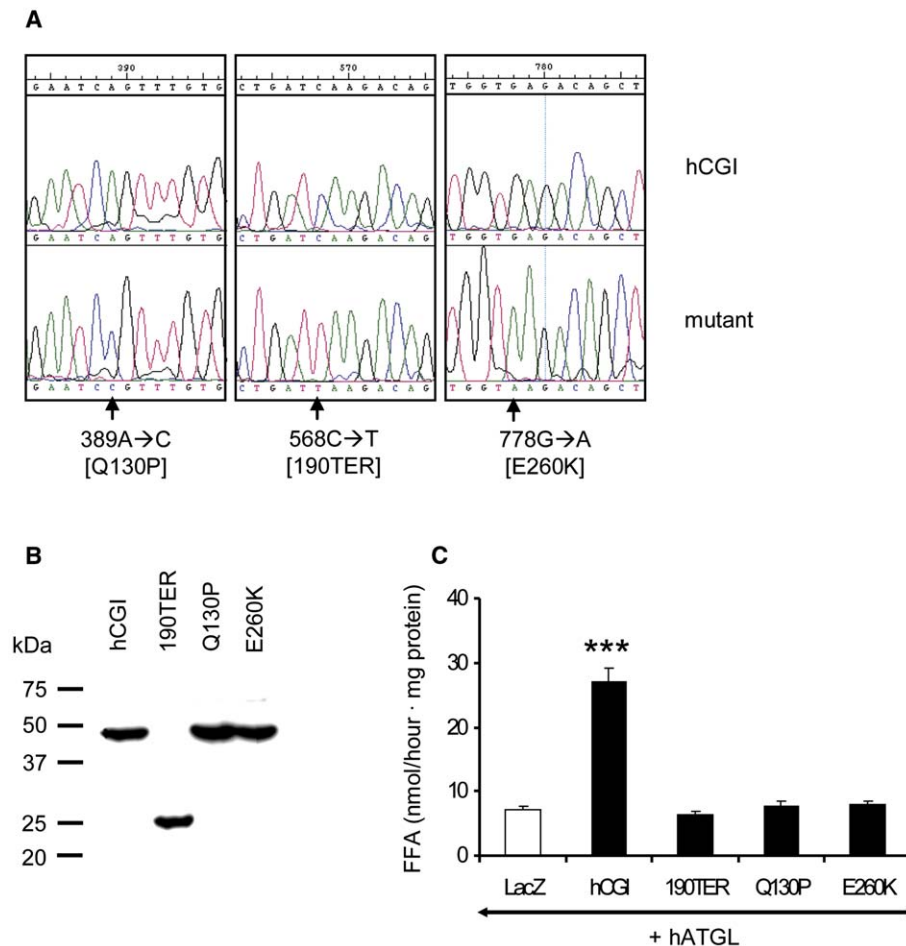
Figure 4. Effect of human CGI-58 (hCGI) and mutant forms of CGI-58 associated with CDS on human ATGL (hATGL) TG hydrolase activity

hATGL, wild-type CGI-58, and mutant versions of hCGI-58 (190TER, Q130P, and E260K) were cloned into the His-tagged pcDNA4/HisMax expression vector. Recombinant proteins were expressed in COS-7 cells.

A) Sequence analysis of cDNAs for hCGI, Q130P, 190TER, and E260K. Nucleotide positions of the mutations are indicated.

B) Cell extracts (20 μg of total protein) were analyzed for the protein expression of hCGI and all mutant versions (190TER, Q130P, and E260K) by Western blotting experiments using anti-His antibody.

C) The capacity of mutant forms of hCGI to activate hATGL was tested in TG hydrolase activity using combined extracts. hATGL was mixed with hCGI at a molar ratio of 1:1 based on immunochemical detection.

Data for TG hydrolase activity assays are presented as mean ± SD and represent three independent experiments (***p < 0.001).

Mutations in CGI-58 that are associated with CDS fail to activate ATGL

Lefèvre et al. (2001) described eight mutations in the human CGI-58 gene that are present in nine families with a confirmed diagnosis of CDS. Of these eight mutations, four were found to be point mutations that, in three cases, led to substitution of a single amino acid. Two of the reported single amino acid variants (Q130P, E260K) were selected in addition to a novel mutation, identified in a cDNA clone we generated from the mRNA of a CDS patient's skin fibroblasts (Igal et al., 1997). Sequencing of this mutant CGI-58 cDNA within the coding region revealed a novel point mutation in nucleotide position 568 (C → T), which results in the formation of a premature TAA stop codon and a truncated protein (190TER). Human mutant cDNAs (Q130P, E260K) were generated by site-directed mutagenesis of wild-type CGI-58 or amplified by PCR (190TER) and ligated into the pcDNA4/HisMaxC expression vector. DNA sequencing confirmed the correct nucleotide substitutions (Figure 4A). Wild-type and mutant hCGI-58 cDNAs were transfected into COS-7 cells and Western blotting analyses of cell lysates revealed a normal MW of 39 kDa for hCGI, Q130P, and E260K (Figure 4B). Due to the premature stop codon, 190TER exhibited a MW of 25 kDa. In TG hydrolysis assays using combined extracts, wild-type hCGI increased the enzyme activity of hATGL 4-fold (Figure 4C). In contrast, all mutant forms of hCGI (190TER, Q130P, and W260K) added at the same 1:1 molar ratio were unable to stim-ulate hATGL activity. Thus, mutations in the human CGI-58 gene with known associations with CDS result in a complete loss of ATGL-activating function.

Expression of functional CGI-58 normalizes the TG content in CDS-HSF

The consequences of dysfunctional activation of ATGL by CGI-58 was assessed by comparing the lipolytic process in normal human skin fibroblasts (HSF) and fibroblasts from a CDS patient (CDS-HSF). As described above (Figure 4), the CGI-58 gene of this patient contains a point mutation and encodes a truncated protein (190TER) with complete loss of function. ATGL and CGI-58 mRNA levels were found to be comparable in both cell lines (Figure 5A). In an attempt to restore lipolysis, CDS-HSF were infected with an adenovirus encoding LacZ, functional CGI-58 (CGI-Ad), or ATGL (ATGL-Ad) (Figure 5B). LacZ infected CDS-HSF contained ∼5-fold more TG than HSF. Expression of functional CGI-58 alone resulted in a 51% reduction of the cellular TG content in CDS-HSF, whereas overexpression of ATGL had no effect. Thus, the abnormal accumulation of TG in CDS-HSF can be reversed by the expression of functional CGI-58.

Functional CGI-58, but not CGI-58 mutants, restores the reduced lipolytic activity in lipid droplets of CDS-HSF

In accordance with previous studies (Williams et al., 1988), TG hydrolase activities were identical in lipid-free cytosolic extracts
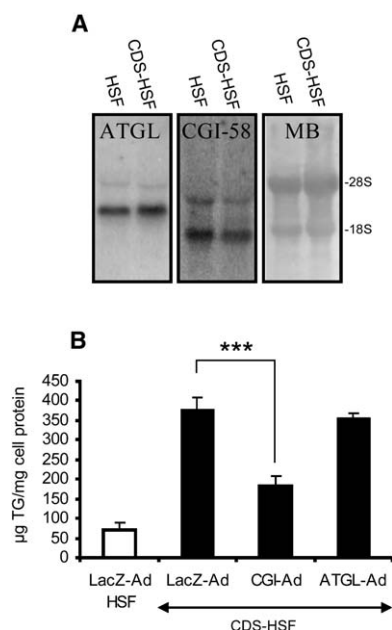
Figure 5. Expression of functional CGI-58 lowers cellular TG accumulation of CDS-derived human skin fibroblasts

A) Northern blotting analysis of total RNA from fibroblasts obtained from a CDS patient (CDS-HSF) and control fibroblasts (HSF). mRNA signal specific for human ATGL or CGI-58 were obtained with ^{32}P-labeled probes. The amount of RNA loaded on the blot was visualized with methylene blue (MB).

B) Effect of adenovirus expression of CGI-58 (CGI-Ad) or ATGL (ATGL-Ad) constructs on TG content of CDS-HSF. For comparison, HSF and CDS-HSF were infected with an adenovirus expressing LacZ. Cellular TG content was determined 2 days after infection.

Data are presented as mean ± SD and represent three independent experiments (***p < 0.001).

of CDS-HSF and HSF (Figure 6A), suggesting that the biochemical defect of CDS is associated with the lipid droplet. To investigate this hypothesis, an in situ lipolysis assay was developed. We reasoned that since ATGL and CGI-58 are both localized to cellular lipid droplets (LD) (Liu et al., 2004; Subramanian et al., 2004; Yamaguchi et al., 2004; Zimmermann et al., 2004), the extent of activation of ATGL by CGI-58 should be measurable in LD "self-digestion" experiments and this parameter was expected to be decreased in LD from CDS-HSF. LD TG were radioactively labeled by cultivating HSF and CDS-HSF in the presence of ^{3}H-9,10-oleic acid. After 24 hr, LD were purified by gradient ultracentrifugation and incubated at 37°C in the presence of bovine serum albumin as FFA acceptor. As predicted, the release of labeled FFA through self-digestion was 66% lower in LD from CDS-HSF than in LD from three HSF lines (Figure 6B). Thus, the LD-associated TG hydrolase activity is markedly impaired in CDS-HSF.

To assess the effect of exogenously added ATGL and CGI-58 to TG hydrolysis in isolated LD, purified mouse proteins were added to radiolabeled LD of HSF or CDS-HSF (Figure 6C). The FFA release from LD of CDS-HSF was significantly lower than from LD of HSF. Addition of purified GST-CGI increased the release of FFA to a similar level in LD of both cell types. In contrast, purified ATGL enhanced the FFA release from HSF more effectively (2.6-fold) than from CDS-HSF, suggesting that ATGL is not fully active on LD which lack functional CGI-58. Addition of both GST-CGI and purified ATGL to LD of HSF and CDS-HSF

elevated FFA release maximally and to a comparable level. Essentially, the same results were obtained when cell extracts containing human CGI-58 and/or human ATGL were incubated with LD from CDS-HSF or HSF (Figure 6D). In contrast, all mutant forms of CGI-58 (Q130P, E260K, and 190TER) failed to induce hATGL-mediated lipolysis in LD from both HSF and CDS-HSF (Figure 6E).

Discussion

The rate of FFA release from adipose tissue in humans and rodents is strongly associated with the development of insulin resistance and type 2 diabetes (Stumvoll et al., 2005), but the mechanisms and regulation of FFA release from stored fat remain insufficiently understood (Boden and Shulman, 2002; Shulman, 2000). The present study provides compelling evidence that CGI-58 is an essential component of the lipolytic system. CGI-58 potently induces the TG hydrolase activity of tissue extracts from adipose tissue and muscle. Increased TG hydrolysis does not result from an intrinsic TG hydrolase activity of CGI-58, but instead from a striking and specific activation of ATGL enzyme activity. HSL activity was not affected by CGI-58. Our findings that overexpression of both ATGL and CGI-58 prevented TG accumulation in COS-7 cells and silencing of ATGL and/or CGI-58 decreased the lipolytic mobilization of stored fat in 3T3-L1 adipocytes provided conclusive evidence that CGI-58 activation of ATGL is physiologically important for the cellular metabolism of neutral lipids in intact cells.

The interaction of cofactors with lipases and activation of TG hydrolysis is not uncommon. Well-known examples include lipoprotein lipase, which requires apolipoprotein CII for optimal activity (Olivecrona and Beisiegel, 1997; Zdunek et al., 2003) against chylomicron and VLDL TG, and pancreatic lipase, which depends on colipase for efficient intestinal digestion of alimentary fat (van Tilbeurgh et al., 1993a; van Tilbeurgh et al., 1993b). Cofactor–lipase binding increases the hydrophobicity of the heterodimeric complex, promoting enhanced substrate binding and efficient enzymatic activity at the water-lipid interphase. ATGL belongs to a new class of lipases containing a "patatin-domain" commonly found in plant acylhydrolases. In the absence of protein structure data for both ATGL and CGI-58, it is difficult to evaluate whether ATGL activation by CGI-58 follows a similar mechanism as has been observed for other TG-hydrolases. However, the observed reversible binding of CGI-58 to LD via perilipin might be of crucial importance in this context (Subramanian et al., 2004; Yamaguchi et al., 2004).

Mutant forms of CGI-58 that cause CDS (Lefèvre et al., 2001) due to single amino acid exchanges or protein truncations are incapable of activating ATGL. Conversely, when functional CGI-58 is expressed in CGI-58-defective fibroblasts of patients with CDS, the abnormal and excessive accumulation of TG is reversed. Several previous studies have provided experimental evidence for a defect in TG hydrolysis or the recycling of acylglycerides to glycerophospholipids in fibroblasts of CDS patients (Igal and Coleman, 1996; Igal and Coleman, 1998; Williams et al., 1991). However, the biochemical defect in CDS and the physiological function of CGI-58 remained unexplained. On the basis of our observations, we propose that the loss of ATGL activation by CGI-58 represents an important dysfunction contributing to the pathogenesis of CDS. Considering the broad expression pattern of ATGL and CGI-58 in many tissues and
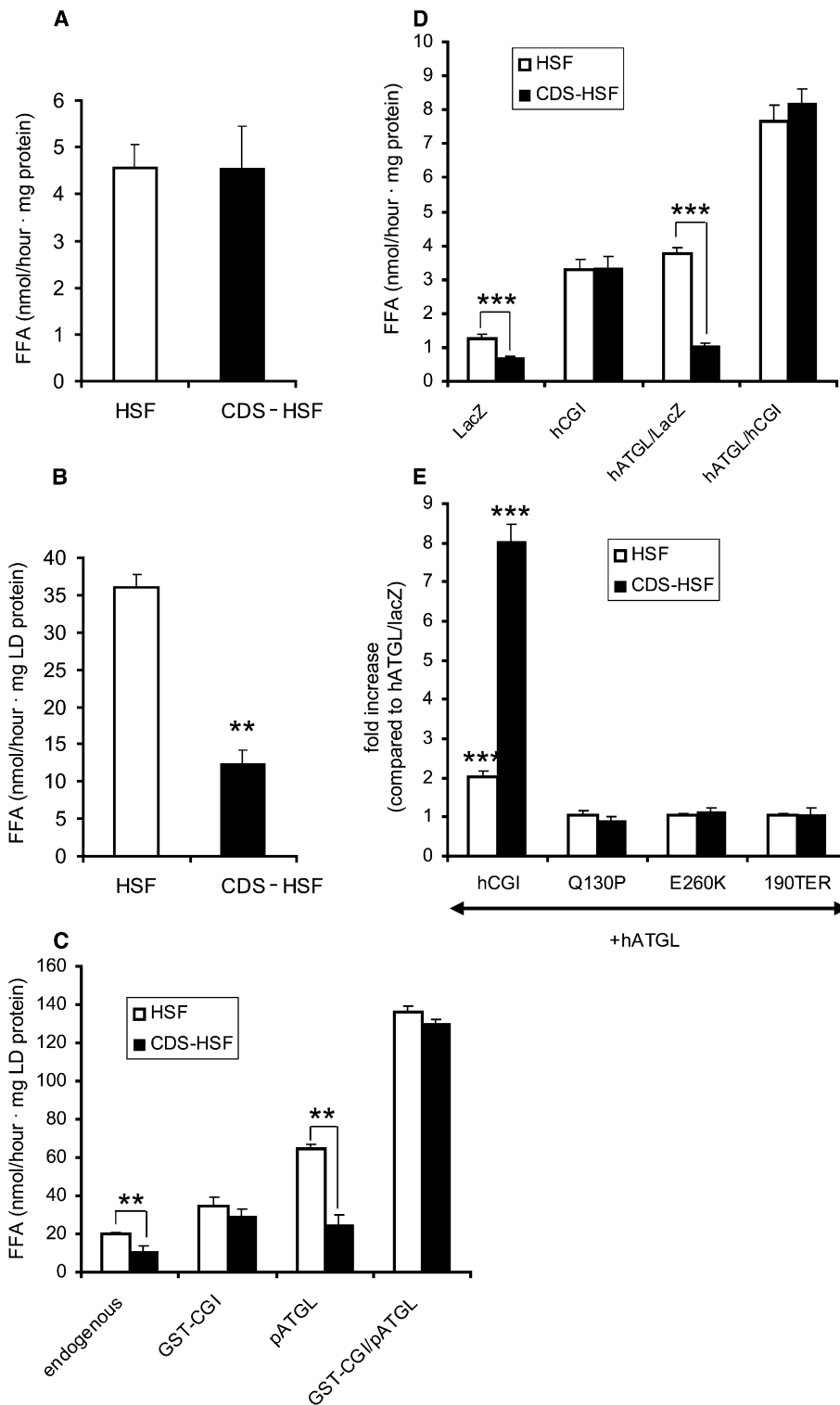
Figure 6. CDS-HSF exhibit reduced lipid droplet-associated TG hydrolase activity, which is restored upon addition of functional CGI-58

A) Cellular TG hydrolase activity of lipid-free cytosolic extracts of CDS-HSF and HSF was determined using a radiolabeled triolein substrate.

B) Lipid droplet-associated TG hydrolase activities of HSF and CDS-HSF. CDS-HSF and HSF were labeled with ^{3}H-9,10-oleate, and radiolabeled LD were purified by gradient ultracentrifugation. Comparable TG loading of cells was achieved by incubating CDS-HSF and HSF in the presence of 0.2 mM and 0.6 mM oleate, respectively. TG hydrolase activity of isolated droplets with comparable protein and TG content was assessed by "self-digestion" in the presence of bovine serum albumin as FFA acceptor.

C) Effect of exogenously added purified mouse CGI-58 (GST-CGI) and ATGL (pATGL) on FFA mobilization of LD derived from HSF and CDS-HSF. LD were incubated in the absence (endogenous activity) or in the presence of GST-CGI or pATGL or in the presence of both proteins and the FFA release was determined.

D) Effect of human hATGL- and hCGI-containing COS-7 cell extracts on FFA mobilization of LD derived from HSF and CDS-HSF. COS-7 cell extracts containing LacZ, hCGI, or mixtures of cell extracts containing hATGL/LacZ or hATGL/hCGI were incubated with ^{3}H-9,10-oleate-labeled LD, and the FFA release was determined. Endogenous activity present on LD was set as blank.

E) Effect of hCGI mutants on hATGL activity using radiolabeled LD as substrate. Cell extracts of COS-7 cells expressing hATGL were mixed with extracts containing LacZ, hCGI, or the mutant versions of CGI-58 (190TER, Q130P, and E260K) and the FFA release was determined. The activity of the extract in the presence of LacZ was arbitrarily set as 1, and the relative increases of TG hydrolase activity are plotted on the y axis.

Data are presented as mean ± SD and represent three independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001).

assuming that ATGL activation by CGI-58 is necessary in all tissues where the enzyme is found, an activation defect would result in the observed accumulation of TG in multiple tissues of CDS patients.

Although ATGL and CGI-58 are coexpressed in many tissues, the relative mRNA expression levels can be quite different. For example, in the liver the concentration of CGI-58 mRNA is high compared to ATGL mRNA, which could not be detected by Northern blotting analysis. This may indicate that CGI-58 activates additional, presently unidentified lipases. Whether dysfunction of these putative ATGL-independent mechanisms also contributes to the clinical heterogeneity in CDS is currently unknown. However, the striking effects on TG accumulation and mobilization in response to variation in CGI-58 and ATGL

expression suggests that, at least in adipocytes, ATGL is the major target for CGI-58-mediated activation of lipolysis. Thus, it is conceivable that a subgroup of CDS patients, particularly individuals where genetic variations in the CGI-58 gene locus are not found, may be affected with mutations in the gene for ATGL.

In addition to the accumulation of fat, the defective ATGL-mediated lipolysis of TG due to insufficient enzyme activation could also account for the defect in the recycling of glycerolipids into glycerophospholipids observed in fibroblasts from CDS patients (Igal and Coleman, 1996). The reaction catalyzed by ATGL is specific for the hydrolysis of the first fatty acid from the TG molecule and thereby generates DG (Zimmermann et al., 2004). The subsequent utilization of DG depends on the metabolic status of the cell. In hormonally stimulated adipocytes under increased energy demand, DG will be instantly hydrolyzed by HSL to generate FFA and glycerol as energy substrates. Alternatively, at times of adequate energy supply, DG can be reesterified to TG by the DG acyltransferase reaction (Buhman et al., 2001) within a futile hydrolysis/reesterification cycle, or utilized for the synthesis of glycerophospholipids as important membrane constituents. Reduced DG formation due to low ATGL activity would impair its utilization for glycerophospholipid synthesis (Igal and Coleman, 1996; Igal and Coleman, 1998).

In conclusion, the activation of ATGL by CGI-58 represents a novel mechanism involved in the lipolytic breakdown of cellular lipid depots. Mutations in CGI-58 as observed in patients affected with CDS result in dysfunctional lipolysis and cellular lipid accumulation. Accordingly, CGI-58 activation of ATGL controls cellular TG catabolism and may represent the dominant genetic defect in CDS.

Experimental procedures

cDNA cloning of recombinant His-tagged proteins

The sequences containing the complete open reading frame of mouse ATGL, HSL, CGI-58, Perilipin A, as well as human ATGL and CGI-58 were amplified by PCR from mouse or human cDNA using Advantage cDNA Polymerase Mix (BD Biosciences Clontech, Palo Alto, CA), respectively. cDNA was prepared from mRNA using SuperScript Reverse Transcriptase protocol (Invitrogen Life Technologies, Carlsbad, CA). The primers were designed to create endonuclease cleavage sites (underlined) for subsequent cloning strategies: mouse ATGL forward 5′-TGGTACCGTTCCCGAGGGAGACCAAGTGGA-3′, mouse ATGL reverse 5′-CCTCGAGCGCAAGGCGGGAGGCCAGGT-3′, mouse HSL forward 5′-TGGTACCTATGGATTTACGCACGATGACACA-3′, mouse HSL reverse 5′-CTCGAGCGTTCAGTGGTGCAGCAGGCG-3′, mouse CGI-58 forward 5′-CGGATCCAAAGCGATGGCGGCGGAGGA-3′, mouse CGI-58 reverse 5′-CCTCGAGTCAGTCTACTGTGTGGCAGATCTCC-3′, mouse Perilipin A forward 5′-CGGTACCGTCAATGAACAAGGGCCCAACC-3′, mouse Perilipin A reverse 5′-CTCTAGATCAGCTCTTCTTGCGCAGCTG-3′, human ATGL forward 5′-CGGGATCCTTTCCCCGCGAGAAGACGTG-3′, human ATGL reverse 5′-CCTCGAGCTCACAGCCCCAGGGCCCC-3′, human CGI-58 forward: 5′-AAGGTACCAGCGGCGGAGGAGGAGGAG-3′, human CGI-58 reverse: 5′-CCTCTAGATCAGTCCACAGTGTCGCAGA-3′.

The PCR products were ligated to compatible restriction sites of the eukaryotic expression vector pcDNA4/HisMax (Invitrogen Life Technologies). A control pcDNA4/HisMax vector expressing β-galactosidase was provided by the manufacturer (Invitrogen Life Technologies).

Cloning of recombinant adenovirus for murine CGI-58 and ATGL expression

For the cloning of mouse ATGL and ATGL-antisense adenovirus constructs, a MluI–ClaI flanked cDNA fragment was amplified by PCR from the cDNA-containing plasmids (described above), using the following primers: mouse ATGL-Ad forward 5′-GCACGCGTCGGACGGCCTCCGAAACC-3′, mouse ATGL-Ad reverse 5′-GCATCGATTAGAAGGCACAGTCGAGGCT-3′, mouse antisense ATGL-Ad forward 5′-GCATCGATTGGAACATCTCATTCGCTGGCT-3′, mouse antisense ATGL-Ad reverse 5′-GCACGCGTGAAGGCCACATTGGTGCAGAAG-3′.

Subsequently, the PCR product was ligated to Mlu I–Cla I digested pAvCvSv34 and the resulting shuttle plasmid was cotransfected with pJM 17 into HEK-293 cells using the calcium phosphate coprecipitation method (Graham and van der Eb., 1973). Cloning of mouse CGI-58 and CGI-58-antisense adenovirus constructs was performed according to manufacturer's instructions using AdEasy XL Adenoviral Vector Sytem (Strategene, La Jolla, CA). The following primers were used: mouse CGI-58-Ad forward 5′-ACCTCGAGCCACCATGAAAGCGATGGCGGCG-3′, mouse CGI-58-Ad reverse 5′-AAAGCTTCTAATGATGATGATGATGATGGTCTACTGTGTGGCAGATCTCC-3′, mouse antisense CGI-58-Ad forward 5′-ACAAGCTTCCACCATGAAAGCGATGGCGGCG-3′, mouse antisense CGI-58-Ad reverse 5′-ACTCGAGCTAGTCTACTGTGTGGCAGATCTCC-3′.

Large-scale production of high-titer recombinant sense and antisense adenoviruses for ATGL and CGI-58 was performed as described (Teng et al., 1994). An adenovirus containing β-galactosidase cDNA (LacZ-Ad) was used as a control.

Generation of site-directed mutagenesis clones

Point mutations were performed by using the GeneTailor Site-Directed Mutagenesis System (Invitrogen Life Technologies). Mutations in CGI-58 (Q130P, E260K) or ATGL (S47A) were introduced at the positions of the coding sequences indicated below using the following primer pairs: Q130P: 389A>C forward 5′-TGCAGAAGAAGTGGAGAATC**C**GTTTGTGGAATCCA-3′, Q130P: 389A>C reverse 5′-GATTCTCCACTTCTTCTGCATCACTGTCAAAC-3′, E260K: 778G>A forward 5′-ATGTGCAGACTCCAAGTGGT**A**AGACAGCTTTC-3′, E260K: 778G>A reverse 5′-ACCACTTGGAGTCTGCACATTACAGTGGTAG-3′, S47A: 139S>A forward 5′-CCACTCACATCTACGGAGCC**G**CGGCAGGGGCGCT-3′, S47A: 139S>A reverse 5′-GGCTCCGTAGATGTGAGTGGCGTTGGCCAC-3′.

Sequence analysis

Sequence analysis of plasmid DNA was performed using the BigDye terminator mixture (Applied Biosystems, Foster City, USA). The PCR products were sequenced on an ABI PRISM 310 Genetic analyzer (Applied Biosystems, Foster City, CA).

Expression of recombinant proteins and preparation of cell extracts

Monkey embryonic kidney cells (COS-7, ATCC CRL-1651) were transfected using Metafectene (Biontex GmbH, Munich, Germany) as described (Zimmermann et al., 2004). For the preparation of cell extracts, cells were collected by trypsination, washed three times with phosphate buffered saline (PBS), and disrupted in buffer A (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 20 µg/ml leupetine, 2 µg/ml antipain, 1 µg/ml pepstatin, [pH 7.0]) by sonication (Virsonic 475, Virtis, Gardiner, NJ). Nuclei and unbroken cells were removed by centrifugation at 1000 × g at 4°C for 5 min. The expression of the His-tagged proteins was detected using Western blotting analysis as described (Zimmermann et al., 2004).

Assay for TG hydrolase activity

For the determination of triglyceride hydrolase activity of various recombinant proteins 40 µg protein of respective cell extracts in a total volume of 100 µl buffer A were incubated with 100 µl substrate in a water bath at 37°C for 60 min. In some cases, recombinant CGI-58 or CGI-58 mutants expressing extracts (20 µg protein) were first mixed with various recombinant protein extracts (20 µg protein) in a total volume of 100 µl buffer A and then incubated with 100 µl substrate. As a control, incubations under identical conditions were performed with LacZ-expressing lysates alone or mixed with various recombinant protein lysates. To ensure linearity of the assay incubation time for reactions with high hydrolytic activities was reduced to 30 or 15 min. Under these conditions in all reactions, less than 10% of the substrate was hydrolyzed. Determination of TG hydrolase activity in cytosolic extracts of various mouse tissues with or without addition of 100 ng GST-CGI was performed as described (Holm and Osterlund, 1999). After incubation, 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 (800 × g, 15 min), the radioactivity in 1 ml of the upper phase was determined by liquid scintillation counting. TG

substrate was prepared by emulsifying 330 μM triolein (40,000 cpm/nmol) and 45 μM phosphatidylcholine/phosphatidylinositol (3:1) in 100 mM potassium phosphate buffer, (pH 7.0), and 5% defatted BSA by sonication.

Labeling and isolation of lipid droplets

HSF obtained from control subjects and CDS-HSF were cultured in DMEM containing 10% FCS. For labeling of TG stores of CDS-HSF, confluent cells were incubated for 24 hr in the presence of 0.2 mM oleate (4 mCi ^{3}H-9,10-oleate/mmol), bound to bovine serum albumin (BSA) at a FFA/BSA molar ratio of 3:1. Comparable TG loading of cells was achieved by incubating CDS-HSF and control cells in the presence of 0.2 mM and 0.6 mM oleate, respectively. For isolation of lipid droplets (LD), cells were collected by trypsination and washed three times with PBS. Thereafter, cells were suspended in buffer A and disrupted by sonication (Virsonic 475, Virtis, Gardiner, NJ). Cell lysates were transferred to SW41 tubes, overlaid with buffer B (50 mM potassium phosphate, [pH 7.4], 100 mM KCl, 1 mM EDTA, 20 μg/ml leupetine, 2 μg/ml antipain, 1 μg/ml pepstatin), and centrifuged in a SW41 rotor (Beckman, Fullerton, CA) (2 hr, 40.000 rpm, 4°C). LD were collected as a white band from the top of the tubes and concentrated by centrifugation (20,000 g, 15 min, 4°C). The underlying solution was removed and LD were resuspended in buffer B by brief sonication. TG and protein contents of LD were determined using commercial reagents (Thermotrace, Thermo Electron Corporation, Victoria, Australia and Bradford, Biorad Laboratories GmbH, Munich, Germany, respectively) and were found to be comparable in both preparations (typically ∼0.5 mg protein/μmol TG for LD of CDS-HSF and HSF). TLC analysis of LD droplets revealed that more than 95% of the radioactivity was present in triglycerides.

Assay for TG hydrolase activity using purified lipid droplets as substrate

To determine LD-associated TG hydrolase activity, 50 μl ^{3}H-9,10-oleate labeled LD at concentration of 400 μM were diluted with 100 μl buffer A and 50 μl delipidated BSA (20%, w/v) and incubated for one hour at 37°C. The release of FFA was determined as described for TG hydrolase activity assays. In some cases, the LD were used as substrate for the determination of TG hydrolase activity in cell extracts or purified proteins.

Accumulation of TG in cultured COS-7 cells expressing various recombinant proteins

COS-7 cells were transfected with murine recombinant His-tagged ATGL, CGI-58, or both ATGL and CGI-58, and as a control LacZ using Metafectene (Biontex GmbH, Munich, Germany) as described (Zimmermann et al., 2004). After 36 hr, cells were incubated with DMEM containing 10% FCS, antibiotics, and 0.5 mM oleate, bound to BSA at a molar ratio of 4:1. After various times the medium was removed and cellular TG content was determined as outlined below.

Adenoviral expression of recombinant proteins in cultured cells

Primary cultures of HSF and CDS-HSF and mouse 3T3-L1 cells (ATCC CL-173) were maintained in Dulbecco's minimal essential medium (DMEM) (GIBCO, Invitrogen Corp., Carlsbad, CA) containing 10% fetal calf serum (FCS) (GIBCO-BRL, Life Technologies, Grand Island, NY) and antibiotics under standard conditions (37°C, 5% CO2).

Two days before adenovirus infection, HSF or CDS-HSF were seeded at a density of 6×10^4 cells per well (6-well plate). HSF were infected with adenovirus constructs expressing murine antisense ATGL (asATGL-Ad) or antisense CGI-58 (asCGI-Ad) and as a control LacZ (LacZ-Ad) with a multiplicity of infection (MOI) of 1600 pfu/cell in DMEM. CDS-HSF were infected with adenovirus constructs expressing ATGL (ATGL-Ad) or CGI-58 (CGI-Ad) and as control LacZ-Ad with a multiplicity of infection (MOI) of 1200 pfu/cell in DMEM. After 2 hr, DMEM containing 10% FCS and antibiotics were added. Then fibroblasts were cultured for 48 hr and cellular TG content was determined as outlined below. Infection efficiency of HSF and CDS-HSF with adenovirus construct encoding LacZ was determined to be ∼40%.

3T3-L1 fibroblasts were induced to differentiate into adipocytes 2 days after confluency, using a standard protocol (Bernlohr et al., 1985). For adenovirus expression, 3T3-L1 adipocytes were infected on day 5 of differentiation with ATGL-Ad, asATGL-Ad, asCGI-Ad, or both asATGL-Ad and asCGI-Ad with a multiplicity of infection of 1600 pfu/cell. For that purpose, the virus suspension was incubated in DMEM containing 0.5 μg/ml polylysine for 100 min.

After addition to the cells and incubation for 24 hr, the medium was removed and cells were incubated for further 24 hr with DMEM containing 10% FCS. In control experiments, 3T3-L1 adipocytes were infected with LacZ-Ad. For determination of FFA and glycerol release, on day 7 of differentiation, 3T3-L1 adipocytes were incubated in DMEM containing 2% fatty acid free BSA with or without 10 μM isoproterenol at 37°C. Aliquots of the medium were collected and the FFA and glycerol content determined as described below. For the preparation of cytosolic lysates of 3T3-L1 adipocytes, on day 5 of differentiation, cells were infected either with adenovirus encoding ATGL-Ad, asATGL-Ad, or as control LacZ-Ad. At day 7 of differentiation, cells were collected by trypsination, washed three times with PBS, and disrupted in buffer A by sonication (Virsonic 475, Virtis, Gardiner, NJ). Cytosolic lysates were obtained by centrifugation at 100,000 × g for 30 min and subjected to TG hydrolase assay.

Preparation and purification of GST-tagged CGI-58

The coding sequence of mouse CGI-58 was cloned into pYex4T-1 vector and transformed into the *S. cerevisiae* BY4742 [Matα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0] strain. Large-scale overexpression of GST-CGI was achieved by maintaining transformed *S. cerevisiae* carrying in YNB-urea containing 0.5 mM CuSO$_4$ to induce copper promoter-driven expression of the fusion cassette. After induction, cells were harvested and protoplasts were generated with zymolyase and disrupted by sonication in the presence of 0.2% NP-40. The supernatant containing the GST-fusion protein was purified using Glutathione-Sepharose beads (GE Healthcare, Piscataway, NJ). Purified GST-CGI was dialyzed overnight with 150 mM KCl, 10 mM potassium phosphate buffer, (pH 7.4), and 0.01% NP-40.

TALON Co^{2+} affinity purification of ATGL

COS-7 cells expressing His-tagged mouse ATGL were lysed in buffer C (25 mM NaPO4, [pH 7.0], 20% glycerol, 2 mM β-mercaptoethanol, 0,01% NP-40) and incubated with TALON Co^{2+} resin (BD Biosciences, Palo Alto, CA). The resin-cell extract suspension was then poured into a micro bio-spin column (Bio-Rad, Hercules, CA) and washed with 10 column volumes of buffer C containing 500 mM NaCl. Recombinant His-tagged ATGL was eluted by a step-gradient of imidazole (200 mM final concentration) in buffer C. For determination of the purity of the protein, fractions were subjected to SDS-PAGE gel electrophoresis and Western blotting analysis.

CGI-58 ELISA

For the detection of interacting proteins, ELISA plates (MaxiSorp, Nalge Nunc Int., Rochester, NY) were coated with 3 μg GST-CGI or GST in buffer D (50 mM Tris, [pH 8.0], 150mM NaCl, 5 mM CaCl2). The wells were blocked with 5% BSA in buffer D and incubated with 50 μg protein/well of COS-7 cell extracts containing His-tagged proteins in 50 mM potassium phosphate buffer (pH 7.0). After washing with buffer D containing 0.05% Tween 20, the mouse anti-His antibody (GE Healthcare) was added in the same buffer containing 0.5% BSA. Subsequent to three further washes, horseradish peroxidase-conjugated anti-mouse antibody (GE Healthcare) was added. After washing three times with buffer D containing 0.05% Tween 20, the absorbance of tetramethyl-benzidine was determined at 450 nm using 620 nm as reference wavelength. The absorbance of GST coated wells was subtracted from that coated with GST-CGI.

Northern blotting analysis

Total RNA from various mouse tissues or HSF and CDS-HSF cells were separated by formaldehyde/agarose gel electrophoresis and blotted onto a Hybond-N+ membrane (GE Healthcare). Mouse and human ATGL were detected with a PstI/XhoI fragment of ATGL and hCGI-58 with the coding region of CGI-58, labeled with [$^{α-32}$P]dCTP. Signals were visualized by exposure to a PhosphorImager Screen (GE Healthcare).

Biochemical analysis

For determination of FFA and glycerol release, 3T3-L1 adipocytes were incubated in DMEM containing 2% fatty acid free BSA with or without 10 μM isoproterenol at 37°C. Aliquots of the medium were collected and the FFA and glycerol content measured with commercial kits (WAKO Chemicals GmbH, Neuss, Germany). For the determination of cellular TG content in COS-7 cells, CDS-HSF or HSF lipids were extracted three times with hexan: isopropanol (3:2; v/v), brought to dryness, solubilized in 0.1% Trition X-100 by

sonication, and TG concentration determined using Infinity Triglycerides reagent (Thermo Electron Corporation, Victoria, Australia). Protein concentrations of cell extracts and cell homogenates were measured with the Bradford protein assay (Bio-Rad Laboratories GmbH, Munich, Germany) and BCA reagent (Pierce Biotechnology, Rockford, IL), respectively, using BSA as standard.

Statistical analysis

Statistical significance was determined by the Student's unpaired t test (two-tailed). Group differences considered significant for $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

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