Adipose triglyceride lipase (ATGL) catalyzes the first step in the hydrolysis of triacylglycerol (TG) generating diacylglycerol and free fatty acids. The enzyme requires the activator protein CGI-58 (or ABHD5) for full enzymatic activity. Defective ATGL function causes a recessively inherited disorder named neutral lipid storage disease that is characterized by systemic TG accumulation and myopathy. In this study, we investigated the functional defects associated with mutations in the ATGL gene that cause neutral lipid storage disease. We show that these mutations lead to the expression of either inactive enzymes localizing to lipid droplets (LDs) or enzymatically active lipases with defective LD binding. Additionally, our studies assign important regulatory functions to the C-terminal part of ATGL. Truncated mutant ATGL variants lacking ~220 amino acids of the C-terminal protein region do not localize to LDs. Interestingly, however, these mutants exhibit substantially increased TG hydrolysis activity in vitro (up to 20-fold) compared with the wild-type enzyme, indicating that the C-terminal region suppresses enzyme activity. Protein-protein interaction studies revealed an increased binding of truncated ATGL to CGI-58, suggesting that the C-terminal part interferes with CGI-58 interaction and enzyme activation. Compared with the human enzyme, the C-terminal region of mouse ATGL is much less effective in suppressing enzyme activity, implicating species-dependent differences in enzyme regulation. Together, our results demonstrate that the C-terminal region of ATGL is essential for proper localization of the enzyme and suppresses enzyme activity.

Adipose triglyceride lipase (ATGL; official gene symbol: PNPLA2, patatin-like phospholipase domain containing 2) is an important triacylglycerol (TG) lipase involved in the mobilization of TG stores (1, 2). The enzyme belongs to a family of patatin domain-containing proteins originally observed in plants (3). The members of this family have been shown to hydrolyze TG, phospholipids, or retinyl ester (4–8).

Defective ATGL function is characterized by systemic TG accumulation in humans (9, 10) and rodents (2). In humans, mutations in both the ATGL gene or the gene for CGI-58 (comparative gene identification-58; also known as α/β-hydrolase fold-containing protein 5, ABHD5) are associated with a rare inherited disorder annotated as neutral lipid storage disease (NLSD) (11). CGI-58 functions as activator protein of ATGL and mutant forms of CGI-58 associated with NLSD completely lose their capability of activating ATGL (12). Although mutations in both ATGL and CGI-58 cause NLSD, the phenotypical appearance of patients is not identical. NLSD caused by defective CGI-58 function (also known as Chanarin-Dorfman syndrome) is clinically characterized by ichthyosis, often associated with mild myopathy and hepatomegaly. Other observed symptoms include ophthalmologic abnormalities, hearing loss, intestinal involvement, short stature, mental retardation, and microcephaly (13–15). In contrast, mutations in ATGL are not associated with ichthyosis. Affected individuals appear to develop a more severe form of myopathy than patients with defective CGI-58 function. Cardiac abnormalities and hepatomegaly have also been described (9, 10). According to these divergent clinical phenotypes, Fischer et al. (9) proposed NLSD with ichthyosis as a name for the subgroup of individuals with mutations in the CGI-58 gene and NLSD with myopathy for individuals with mutations in the ATGL gene.

Because naturally occurring mutations in human genes offer a unique opportunity to study the structure-function relationship of enzymes, we investigated the functional defects of mutations in the ATGL gene causing NLSD. Our results identify the biochemical basis of the known genetic defects and assign an important function to the previously uncharacterized C-terminal region of the protein, which affects enzyme activity and mediates LD binding of the enzyme.
Structure-Function Relationship of ATGL

EXPERIMENTAL PROCEDURES

cDNA Cloning of Recombinant Proteins—The sequences containing the complete open reading frame of human ATGL (hATGL) and CGI-58 were amplified by PCR from human cDNA using Advantage cDNA polymerase mix (BD Biosciences Clontech, Palo Alto, CA). cDNA was prepared from mRNA using SuperScript Reverse Transcriptase protocol (Invitrogen). The primers were designed to create endonuclease cleavage sites (underlined) for subsequent cloning strategies: hATGL HisMaxC forward, 5’-CCCGTCTGACAGCCCGAGCCGGACAGC-3’; hATGL HisMaxC reverse, 5’-CCCGTCTGACAGCCCGAGCCGGACAGC-3’; hATGL_YPF-C1 forward, 5’-ATCTCGAGGAGCATGCTGCTG-3’; hATGL_YPF-C1 reverse, 5’-GGATCCGCTGCGAGCAGCAG-3’; hATGL bp799 forward, 5’-GAACATCTGTCTGCAGGACAGCTC-3’; and mATGL289_HisMaxC reverse, 5’-CTGCCGGGAGA-3’.

The PCR products were ligated to compatible restriction sites of the eukaryotic expression vector pcDNA4/HisMaxC (Invitrogen). The primers were designed to create endonuclease cleavage sites (underlined) for subsequent cloning strategies: hATGL HisMaxC forward, 5’-CCCGTCTGACAGCCCGAGCCGGACAGC-3’; hATGL HisMaxC reverse, 5’-CCCGTCTGACAGCCCGAGCCGGACAGC-3’; hATGL bp799 forward, 5’-GTGGATCCGCTGCGAGCAGCAG-3’; hATGL bp799 reverse, 5’-GGGCGGGCGGGGGCAACGCCAGCAAGGGG-3’.

The PCR products were sequenced to confirm the correct sequences of the coding sequences indicated below using the following mutagenesis system (Invitrogen). Mutations in human ATGL (P195L, FS270, FS282, and Q289X) were introduced at the positions specified below. cDNA was prepared from cDNA using Advantage cDNA polymerase mix (BD Biosciences Clontech, Palo Alto, CA). A control pcDNA4/HisMax vector expressing lactosidase was provided by the manufacturer (Invitrogen).

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

Expression of Recombinant Proteins and Preparation of Cell Extracts—Monkey embryonic kidney cells (Cos-7, ATCC CR-L-1651) were cultivated in DMEM (Invitrogen) containing 10% fetal calf serum (Sigma-Aldrich) under standard conditions (37 °C, 5% CO2). The cells were transfected with 1 µg DNA complexed to Metafectene (Biontex GmbH, Munich, Germany) in serum-free DMEM. After 4 h the medium was replaced by regular growth medium supplemented with 10% fetal calf serum. For the preparation of cell extracts, the cells were washed with PBS, collected using a cell scraper, and disrupted in buffer A (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 20 µg/ml leupeptin, 2 µg/ml antipain, 1 µg/ml pepstatin, pH 7.0) by sonication (Virtis 475, Virtis, Gardner, NJ). The nuclei and unbroken cells were removed by centrifugation at 1,000 × g, 4 °C for 10 min. Protein concentration of cell lysates was determined with Bio-Rad protein protein assay according to the manufacturer’s protocol (Bio-Rad 785) using BSA as standard. The expression of the His-tagged proteins was detected by Western blotting analysis as described (1).

Assay for TG Hydrolase Activity—For the determination of TG hydrolase activity of various recombinant proteins, 10–40 µg of protein of respective cell extracts in a total volume of 100 µl of buffer A were incubated with 100 µl of substrate in a water bath at 37 °C for 60 min. As a control, incubations under identical conditions were performed with LacZ-expressing lysates alone or mixed with various recombinant protein lysates. 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 x g, 15 min), the radioactivity in 1 ml of the upper phase was determined by liquid scintillation counting.

Stimulation of ATGL was achieved by co-incubation of the enzyme either with purified murine GST-CGI-58 (GST-CGI) or Cos-7 lysates expressing His-tagged human CGI-58 (hCGI-58). GST-CGI and hCGI-58 were added at a concentration leading to maximal stimulation of ATGL (12, 17) (300 ng/assay for GST-CGI; equimolar concentrations of hCGI-58 and ATGL based on the expression levels of the His-tagged proteins). TG substrate was prepared by emulsifying 330 µCi 3H-9,10-oleate (40,000 cpm/nmol) and 45 µM phosphoryldihydroxycholine/phosphatidylinositol (3:1) in 100 mM potassium phosphate buffer, (pH 7.0) by sonication (18) and adjusted to 5% defatted BSA.

Labeling and Isolation of LD—Human skin fibroblasts of Patient 2 (FS282 mutation) were cultured in DMEM containing 10% fetal calf serum. For radioactive labeling of TG, confluent cells were incubated for 20 h in the presence of 0.2 mM oleate (4 µCi 3H-9,10-oleate/nmol) complexed to BSA at a FFA/BSA molar ratio of 3:1. For isolation of LD, the cells were washed with PBS and collected using a cell scraper. Thereafter, the cells were suspended in buffer B and disrupted by sonication (Virtis 475, Virtis, Gardner, NJ). The 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 leupeptin, 2 µg/ml antipain, 1 µg/ml pepstatin), and centrifuged in a SW41 rotor (Beckman, Fullerton, CA) (2 h, 100,000 × g, 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, Bio-Rad Laboratories GmbH, Munich, Germany, respectively).

**Assay for TG Hydrolyase Activity Using Purified LD as Substrate**—For the determination of TG hydrolyase activity of various recombinant proteins, 40 μg of protein of respective cell extracts were incubated with 25 nmol 3H-9,10-oleate labeled LD (1,660 cpm/nmol TG) and 5% defatted BSA in a total volume of 200 μl. The reaction was incubated for 1 h at 37 °C. The release of FFA was determined as described for TG hydrolyase activity assays using an artificial substrate.

**Cellular Localization of ATGL Mutants**—Cos-7 cells were seeded on glass coverslips in 6-well dishes (1.5 × 10^5 cells/well) and transfected with YFP-tagged human ATGL (hATGL) and ATGL mutants. 24 h after transfection, the cells were incubated for 20 h in regular growth medium supplemented with oleic acid (400 μM) complexed to fat-free BSA. Lipid droplets were stained by incubating cells with 15 μg/ml Bodipy® 558/568 C_{12} (Invitrogen) in DMEM for 2 h. The cells were washed three times with 1 × PBS before mounting them on a Nipkow®-based array confocal laser scanning microscope (19). The array confocal laser scanning microscope was built on a Zeiss Axiovert 200M (Zeiss Microsystems, Jena, Germany) equipped with VoxCell Scan® (VisiTech, Sunderland, UK), a 150-milliwatt Argon laser (Laser Physics; West Jordan, UT), and a 30-milliwatt 405-nm laser diode (VisiTech). Single cells displaying a clear fluorescence were selected to acquire three-dimensional stacks (with a z-distance of 100 nm) using the emission filter 558/568 (Omega Optical, Brattleboro, VT). Bodipy® 558/568 C_{12} fluorescence was excited at 514 nm and detected at 570 nm using the emission filter 558/568 (Omega Optical, Brattleboro, VT).

For quantification of fluorescence signals, five LDs were randomly selected in single cells, and the average fluorescence intensity for YFP-tagged ATGL variants was obtained along a circular line at the edge of each droplet. In analogy, the average intensity of the respective circular line at least 2 μm away from the lipid droplets was extracted to measure LD-free cytoplasm. Differences of the subcellular localization different ATGL constructs were expressed as the ratio between the average fluorescence intensities at LDs and the average intensities in the cytoplasm. All of the image analyses were performed using Metamorph 5.0 (Universal Imaging, VisiRon Systems, Puchheim, Germany) (20).

**Isolation of Lipid Droplets for Western Blot Analysis**—24 h after transfection, Cos-7 cells were incubated for 20 h in regular growth medium supplemented with oleic acid (400 μM) complexed to fat-free BSA (molar ratio 3:1). Thereafter, the cells were washed with PBS and collected using a cell scraper in buffer A containing 1 mM phenylmethysulfonyl fluoride and 1 mM EDTA. The cells were disrupted by sonication (Virsionc 475, Virtis, Gardiner, NJ), transferred to SW41 tubes, and centrifuged as described above. Proteins of the LD fraction were subjected to SDS-PAGE and Western blotting analysis using an anti-His antibody (GE Healthcare).

**CGI-58 ELISA**—For the detection of interacting proteins, ELISA plates (MaxiSorp, Nalgene Nunc Int., Rochester, NY) were coated with 3 μg of GST-CGI in buffer C (50 mM Tris, pH 8.0, 150 mM NaCl). The wells were blocked with 5% BSA in buffer C and incubated with 50 μg of protein/well of Cos-7 cell extracts in 50 mM potassium phosphate buffer, pH 7.0, containing equimolar concentrations of His-tagged proteins. After washing with buffer C 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 C containing 0.05% Tween 20, the absorbance of tetramethyl-benzidine was determined at 450 nm using 620 nm as reference wavelength.

**Biochemical Analysis**—TG concentration was determined using Infinity Triglycerides reagent (Thermo Electron Corporation). Protein concentrations of cell extracts were measured with the Bradford protein assay (Bio-Rad) and BCA reagent (Pierce), 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 (***)

**RESULTS**

**Structure of Wild-type and Mutant Human ATGL**—Previous sequence analysis and three-dimensional structural comparisons with related proteins of the PNPLA family showed that the N-terminal part (residues 1–251) of ATGL belongs to the class of α/β proteins containing a patatin domain of a three-layer (α/β/α) sandwich architecture (residues 10–178). In addition to the name giving plant protein patatin, Pat17 (21), this homologous superfamily (CATH code 3.40.1090.10) also contains the catalytic domain of human cytosolic phospholipase A2 (cPLA2) (22) with known three-dimensional structure. In these proteins, the hydrolytic reaction is mediated through a catalytic serine-aspartate dyad (Ser^{47}–Asp^{215} in Pat17, Ser^{228}–Asp^{548} in cPLA2, and Ser^{47}–Asp^{166} in ATGL (predicted), with the nucelophilic serine located within a GXSXG motif typically found in lipases of the α/β-hydrolyase fold family (Fig. 1a). The C-terminal part (residues 250–504) is expected to consist mostly of α-helical and loop regions. A hydrophobic stretch (amino acid 315–360) potentially represents a lipid-binding region.

Fischer et al. (9) described four different mutations associated with NLD with myopathy (Fig. 1a). Patient 1 was reported as compound heterozygote. One mutation led to an amino acid exchange within the α/β-fold at position 195 (P195L). A single base pair deletion on the second allele resulted in a frameshift at position 270 (FS270) leading to the expression of a protein with 319 amino acids (aa). Patient 2 exhibited a homozygous single base pair deletion resulting in a frameshift at position 282 (FS282) and the expression of a protein with 319 aa. Patient 3
Structure-Function Relationship of ATGL

(a) hATGL and mutant ATGL variants P195L, FS270, FS282, and Q289X. a, domain organization of wild-type and mutant ATGL variants. b, Western blot analysis of His-tagged proteins expressed in Cos-7 cells using an anti-His Antibody. c, TG hydrolase activity in cell lysates expressing ATGL or mutant ATGL using an artificial triolein substrate. d, TG hydrolase activity of hATGL and mutant ATGL using purified LDs as substrate. The measurements were performed in the absence (basal) and in the presence of human CGI-58 (hCGI-58). The other mutants again exhibited increased activity. Based on the expression levels of the respective His-tagged proteins, FS270, FS282, and Q289X were 2-, 4.5-, and 8.6-fold more active than hATGL, respectively (Fig. 1c). A comparable increase in TG hydrolase activity was also observed in the presence of hCGI-58 (1.7-, 5.4-, and 11.6-fold for FS270, FS282, and Q289X, respectively, compared with CGI-58-stimulated hATGL). Thus, ATGL mutants lacking the C-terminal region exhibit increased in vitro TG hydrolase activity compared with the full-length enzyme.

TG Hydrolase Activity of ATGL Mutants Using Lipid Droplets as Substrate—To investigate whether ATGL mutants are also active against a physiologically more relevant substrate, we used purified LD as substrate in TG hydrolase assays (Fig. 1d). Similar as observed with the artificial substrate, P195L exhibited decreased activity in the absence and in the presence of CGI-58. The other mutants again exhibited increased activity. Based on the expression levels of the respective His-tagged proteins, FS270, FS282, and Q289X were 2.0-, 7.6-, and 24.6-fold more active than hATGL. In the presence of hCGI-58, TG hydrolase activity was increased 1.3-, 5.0-, and 22.3-fold for hATGL and P195L, respectively (Fig. 1d). These results indicate that ATGL mutants lacking the C-terminal region exhibit increased TG hydrolase activity compared with the full-length enzyme.

Cellular Localization of ATGL Mutants—Next we determined whether ATGL mutations affect the cellular localization of ATGL. Cos-7 cells were transfected with YFP-tagged wild-type and mutant ATGL. The YFP-tagged proteins exhibited similar TG hydrolase activity compared with the His-tagged constructs, suggesting that the YFP tag did not interfere with the enzymatic function (data not shown). As shown in Fig. 2a, hATGL and P195L mainly localized to LD and were only barely detectable in the cytosol. In contrast, ATGL mutants FS270, FS282, and Q289X were located in the cytosol and barely detectable around LDs. To compare LD association of hATGL and mutants, we quantified fluorescent signals in the LD-free cytosolic fraction (F-cytosol) and around lipid droplets (F-LD). Compared with hATGL and P195L, mutants FS270, FS282, and Q289X exhibited a substantially decreased ratio of F-LD/F-cytosol again indicating defective LD binding (Fig. 2b). The quantification of fluorescent signals of whole cells (Fig. 2c) as well as Western blotting analysis of ATGL constructs (Fig. 2d) revealed that wild-type and mutant enzymes were expressed at comparable levels, suggesting that the localization was homozygous for a nonsense mutation, which led to the expression of a protein with 289 amino acids (Q289X).

TG Hydrolase Activity of ATGL and ATGL Mutants—To investigate functional defects caused by different mutations, Cos-7 cells were transfected with expression vectors encoding His-tagged wild-type ATGL or the four mutant versions of the enzyme described above. Expression of proteins and their molecular weights were determined by Western blotting analysis (Fig. 1b). For the determination of TG hydrolase activity, cytoplasmic lysates of Cos-7 cells were incubated with an artificial triolein substrate emulsified with phospholipids (Fig. 1c). Compared with wild-type human ATGL (hATGL), the allele with a single missense mutation in amino acid position 195 (P195L) exhibited reduced TG hydrolase activity in the absence (−45%, p = 0.06) and in the presence (−87%) of human CGI-58 (hCGI-58). In contrast and quite unexpectedly, all of the deletion mutants of ATGL exhibited increased activity. Based on the expression levels of the His-tagged proteins, FS270, FS282, and Q289X were 2-, 4.5-, and 8.6-fold more active than hATGL, respectively (Fig. 1c). A comparable increase in TG hydrolase activity was also observed in the presence of hCGI-58 (1.7-, 5.4-, and 11.6-fold for FS270, FS282, and Q289X, respectively, compared with CGI-58-stimulated hATGL). Thus, ATGL mutants lacking the C-terminal region exhibit increased in vitro TG hydrolase activity compared with the full-length enzyme.

FIGURE 1. Domain organization and TG hydrolase activity of hATGL and mutant ATGL variants P195L, FS270, FS282, and Q289X. a, domain organization of wild-type and mutant ATGL variants. b, Western blot analysis of His-tagged proteins expressed in Cos-7 cells using an anti-His Antibody. c, TG hydrolase activity in cell lysates expressing ATGL or mutant ATGL using an artificial triolein substrate. d, TG hydrolase activity of hATGL and mutant ATGL using purified LDs as substrate. The measurements were performed in the absence (basal) and in the presence of human CGI-58 (hCGI-58), as well as Western blotting analysis of ATGL constructs. The data are presented as the means ± S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
FIGURE 2. Localization of wild-type and mutant ATGL variants in Cos-7 cells. The cells were transfected with an expression vector encoding YFP-tagged constructs. To increase TG storage, the cells were incubated with 0.4 mM oleic acid complexed to albumin for 20 h. Neutral lipids in cells were stained with Bodipy® 558/568 C12 for 2 h. Cellular localization of YFP-ATGL proteins was determined by Nipkow®-based array confocal laser scanning microscopy. a, left panels show immunofluorescent signal of single cells and a portion of a single cell (green), Bodipy staining is shown in red, and the right panels show merged images. b, fluorescence intensities for YFP-tagged constructs were measured along a circular line at the edge of each droplet and in LD-free cytoplasm. Differences in subcellular localization are expressed as the ratio between the fluorescence intensities at LDs and intensities in the cytoplasm. In each cell, five LDs and five LD-free areas were randomly selected. The data are presented as the means ± S.D. of 20 single cells. c, fluorescence intensities of whole cells. d, Western blotting analysis of YFP-tagged ATGL constructs. The proteins were detected in cytoplasmic fractions (1000 g of supernatant, 10 μg of protein/lane) using a polyclonal antibody raised against human ATGL (Cell Signaling). e, association of ATGL variants with purified LDs. Cos-7 cells were transfected with an expression vector encoding His-tagged ATGL constructs and incubated in the presence of 0.4 mM oleic acid complexed to albumin for 20 h. Thereafter, LD were isolated by ultracentrifugation, and LD-associated proteins were subjected to Western blotting analysis (2 μg of protein/lane) using an anti-His antibody. The Coomassie-stained blot is shown as loading control.
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(a) Structure of ATGL

(b) Activity of ATGL in Basal Conditions

(c) Activity of ATGL with CGI-58

(d) Activity of hATGL and mATGL in Basal Conditions

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of ATGL variants is not affected by different expression levels. Similar results as shown for YFP-tagged proteins were obtained in cell fractionation experiments using His-tagged constructs. As shown in Fig. 2e, signals for truncated ATGL mutants detected in isolated LDs were substantially decreased compared with hATGL, whereas P195L exhibited increased LD binding. Together, our data suggest that the C-terminal region is essential for proper localization of ATGL.

Comparison of TG Hydrolase Activity of Human and Mouse ATGL—Human ATGL was reported to possess low in vitro TG hydrolase activity compared with the mouse ortholog (mATGL) or hormone-sensitive lipase (12, 23). Sequence comparison of hATGL and mATGL reveals 84% identity and 87% homology. The N-terminal 266 aa of human and murine orthologs exhibit 96% homology. Stretches with low similarity can be found in the C-terminal part of the enzymes (indicated in red, Fig. 3a), which might be causal for the reported low TG hydrolase activity of hATGL. To compare the activity of human and murine orthologs in the presence and in the absence of their C-terminal regions, full-length enzymes and truncated ATGL variants were expressed in Cos-7 cells. Based on the expression levels of the His-tagged constructs, we found that mATGL is 8.0- and 9.2-fold more active than hATGL in the absence and presence of purified CGI-58, respectively (Fig. 3b). In comparison, Q289X exhibited increased TG hydrolase activity compared with mATGL (2.1- and 1.3-fold in the absence and in the presence of CGI-58, respectively). To investigate whether the C-terminal region also affects the activity of mATGL, we generated a construct encoding the N-terminal 289 aa of mATGL (m289X). In contrast to the human enzyme, truncation of mATGL did not affect basal activity (Fig. 3c). In the presence of CGI-58, m289X was 2-fold more active than the full-length enzyme. These data suggest that the activity of mATGL is influenced to a much lower extent by its C-terminal region compared with the human ortholog.

TG Hydrolase Activity of Chimeric Enzymes—To investigate whether the C-terminal region of hATGL is capable of suppressing the activity of mATGL, we produced a chimeric enzyme (mN/hC-ATGL) consisting of the N-terminal 266 aa of mATGL and the C-terminal part of hATGL (aa 267–504). Compared with mATGL, mN/hC-ATGL showed markedly decreased TG hydrolase activity in the absence and in the presence of CGI-58 (~83% and ~90%, respectively; Fig. 3d). In contrast, a chimeric enzyme consisting of the N-terminal region of hATGL and the C-terminal part of mATGL (hN/mC-ATGL) exhibited increased activity compared with hATGL (4-fold \(p = 0.07\) and 7.2-fold in the absence and in the presence of CGI-58, respectively). Thus, the comparatively low activity of hATGL can largely be explained by the activity-suppressing character of the human C-terminal region.

Interaction of ATGL Variants with CGI-58—To compare the interaction of hATGL, mATGL, truncated, and chimeric proteins, we determined the binding of the His-tagged enzymes to GST-CGI, which was immobilized on ELISA plates as described (12). Compared with \(\beta\)-galactosidase (LacZ), which was used as negative control, hATGL and mATGL exhibited a significant increase in CGI-58 binding (Fig. 4a). hATGL only barely interacted with GST-CGI compared with mATGL (~68% after subtraction of the LacZ control). hN/mC-ATGL showed increased binding compared with hATGL (2.5-fold). In contrast, mN/hC-ATGL exhibited reduced binding compared with mATGL (~59%; Fig. 4b). The truncated enzymes Q289X and m289X exhibited 2.5- and 1.6-fold increased GST-CGI binding in comparison with their respective wild-type enzymes (Fig. 4b). Together, these data suggest that ATGL interacts with CGI-58 in its N-terminal region. The C-terminal region of the enzyme interferes with CGI-58 binding. As observed in activity assays, the suppressive effect of the C-terminal region is more pronounced in the human ortholog compared with mATGL.
Activity of P195L and Interaction with CGI-58—P195L exhibits reduced TG hydrolase activity compared with the wild-type enzyme (Fig. 1, c and d). To investigate whether this mutation affects the activity of the enzyme or its interaction with CGI-58, we generated a construct encoding the N-terminal 289 aa of P195L (P195L/289X). As shown in Fig. 5a, truncation of P195L did not increase enzyme activity in the absence or in the presence of CGI-58. Interaction studies with GST-CGI revealed that P195L and P195L/289X are capable of binding CGI-58 (Fig. 5b), suggesting that the P195L amino acid substitution affects the enzymatic activity of ATGL rather than its interaction with CGI-58.

**DISCUSSION**

Excess FFA are stored in the form of TG in cytosolic lipid droplets. Although many cell types are capable of storing TG, most of FFA are deposited in adipose tissue. In times of starvation or in periods of increased energy demand, adipocytes release FFA into the circulation to provide the body with energy. The concentration of circulating FFA is determined by a balance between TG synthesis and hydrolysis in adipose tissue. An increased net release may result in elevated FFA levels in the body and is shown as inset b, binding of His-tagged β-galactosidase (LacZ), P195L, and P195L/289X (expressing the N-terminal 289 aa of P195L) to CGI-CGI-coated ELISA plates. The experiments were performed with Cos-7 cell lysates containing equimolar concentrations of the His-tagged constructs as described in Fig. 4. The measurements were performed in triplicate and are representative for two independent experiments. The data are presented as the means ± S.D. ***p < 0.001.

ATGL performs the first step in the hydrolysis of TG generating FFA and diacylglycerol. In humans and mice, defective ATGL activity is associated with systemic TG accumulation, indicating a function of the enzyme in multiple tissues (2, 9). In this study, we analyzed the functional defects caused by mutations in the ATGL gene that are associated with NLSD. Our study demonstrates that NLSD may be caused by mutations leading to the expression of inactive ATGL or active lipases with reduced lipid droplet binding. The N-terminal region of ATGL is predicted to adopt an α/β/α sandwich structure containing a patatin domain and a GXSXG consensus motif with the active serine. The P195L mutation led to the substitution of a single amino acid within the lipase-typical α/β-structure, resulting in substantially decreased lipase activity. The YFP-tagged P195L mutant was predominantly localized on lipid droplets, demonstrating that this mutation did not decrease the lipid droplet binding of ATGL. The P195L mutation is located outside the amino acid stretch of ATGL that shows similarity to patatin (aa 10–178 in ATGL), and it can be expected that the architecture of the predicted catalytic site comprised of Ser47 and Asp166 of ATGL remains intact. However, our data show this aa substitution drastically affects the catalytic function of the enzyme, whereas the interaction with CGI-58 seems unaffected.

In contrast to P195L, all of the mutations that left the N-terminal part of ATGL intact were enzymatically active and stimulated by CGI-58. Moreover, truncated mutants missing most of the C-terminal region were up to 20-fold more active than full-length ATGL, suggesting that the C-terminal region is involved in the regulation of enzyme activity. The activity of mutants was tested using an artificial substrate and purified LDs containing numerous proteins that might positively or negatively affect lipolysis. ATGL mutants were active against both substrates, demonstrating that they are also capable of hydrolyzing TG in the presence of the LD-associated proteins. Together, these observations would predict rather functional substrate binding and enhanced lipolysis than defective lipolysis and TG accumulation as observed in tissues and cultured cells of NLSD patients (9). However, in accordance with the NLSD phenotype, studies with YFP-tagged proteins revealed that the cellular appearance of these mutants is predominantly cytosolic because of defective LD binding. Thus, the *in vitro* activity of ATGL does not predict the capacity of the enzyme to hydrolyze TG *in vivo*. Presumably, additional yet unidentified factors control the targeting of the enzyme to the lipid droplet and its activity. The C-terminal region of ATGL apparently possesses two functions: (i) a negative regulatory function affecting the activity of ATGL and (ii) a domain or binding site necessary for efficient substrate binding *in vivo*. ATGL and hormone-sensitive lipase (HSL) are the major enzymes in adipose triglyceride catabolism. Together, these...
enzymes are responsible for more than 95% of the TG hydrolase activity present in adipocytes (17). Both enzymes are activated by signals that raise the cAMP levels and activate protein kinase A (PKA). PKA phosphorylates HSL and the LD-associated protein perilipin A. This process leads to the translocation of HSL from the cytosol to the LD where the enzyme gains access to the TG substrate (26). In contrast to HSL, ATGL is not a target for PKA-mediated phosphorylation (1). In adipocytes, ATGL is present on lipid droplets and in the cytosol. This distribution pattern does not markedly change in lipolytically stimulated cells, which excludes an activation mechanism based on the translocation of the enzyme (1, 27). However, recent observations suggest that the perilipin-adipophilin-Tip47 family proteins adipocyte differentiation-related protein and perilipin A are involved in the regulation of ATGL activity. Listenberger et al. (28) showed that overexpression of adipocyte differentiation-related protein reduces ATGL binding to lipid droplets in various cell lines. In adipocytes, current data suggest that PKA regulates ATGL-mediated lipolysis by an indirect process that involves CGI-58 and perilipin A. In the basal state, CGI-58 is bound to LDs and interacts with perilipin. In the activated state, the phosphorylation of perilipin leads to the release of CGI-58 (27, 29, 30). It was hypothesized that ATGL activity is controlled by CGI-58 independent of the presence of perilipin A and thus could represent an activation event in tissues where perilipin A is not expressed.

In addition, ATGL is phosphorylated at two positions in the C-terminal region (Ser404 and Ser428) (16). To date, it is not known whether phosphorylation events affect the activity of the enzyme or its localization. Our data clearly indicate that the molecular mechanisms regulating ATGL activity could involve additional regulatory steps. Apparently, most of the activity of the enzyme is masked by its C-terminal region, and full activity can be detected only in proteins lacking the C-terminal part. The increased binding of the truncated ATGL to CGI-58 suggests that the C-terminal region controls the access of CGI-58 to ATGL. We propose that conformational changes in the C-terminal region are necessary to unmask the activity of the human enzyme, which might be induced by phosphorylation events and/or chaperone activity. It is reasonable to assume that such a mechanism can control ATGL activity and the activation of the enzyme by CGI-58 independent of the presence of perilipin A and thus could represent an activation event in tissues where perilipin A is not expressed.

In comparison with the full-length human enzyme, mouse ATGL is severalfold more active in hydrolyzing TG. Truncation of both human and mouse ATGL at position 289 increased enzyme activity, implicating that the activity of both orthologs is suppressed by their C-terminal region. However, the extent of enzyme activation was much higher in the human ortholog implicating species-dependent differences in enzyme regulation. Studies with chimeric enzymes revealed that an exchange of the C-terminal regions between mouse and human orthologs suppresses the activity of mATGL and increases the activity of hATGL. Thus, specifically in humans ATGL activity is efficiently suppressed by the C-terminal region of the enzyme.

Together, studies with mutant ATGL revealed that the C-terminal region is essential for proper localization of the enzyme and possesses a negative regulatory function. The higher in vitro activity of truncated human ATGL and its increased interaction with CGI-58 indicates that this region controls ATGL activity by interfering with CGI-58 binding and enzyme activation. We propose that ATGL activity is regulated by C-terminal events that increase the interaction of CGI-58 and ATGL. These processes might include phosphorylation processes at the C-terminal region and/or interaction with unidentified regulatory proteins.

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