Original Article

Identification of a Novel Member of the Carboxylesterase Family That Hydrolyzes Triacylglycerol

A Potential Role in Adipocyte Lipolysis

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Molecular mechanisms underlying lipolysis, as defined by mobilization of fatty acids from adipose tissue, are not fully understood. A database search for enzymes with α/β hydrolase folds, the GXSXG motif for serine esterase and the His-Gly dipeptide motif, has provided a previously unannotated gene that is induced during 3T3-L1 adipocytic differentiation. Because of its remarkable structural resemblance to triacylglycerol hydrolase (TGH) with 70.4% identity, we have tentatively designated this enzyme as TGH-2 and the original TGH as TGH-1. TGH-2 is also similar to TGH-1 in terms of tissue distribution, subcellular localization, substrate specificity, and regulation. Both enzymes are predominantly expressed in liver, adipose tissue, and kidney. In adipocytes, they are localized in microsome and fatcake. Both enzymes hydrolyzed *p*-nitophenyl butyrate, triolein, and monoolein but not diolein, cholesteryl oleate, or phospholipids; hydrolysis of short-chain fatty acid ester was 30,000-fold more efficient than that of long-chain fatty acid triacylglycerol. Fasting increased the expression of both genes in white adipose tissue, whereas refeeding suppressed their expression. RNA silencing of TGH-2 reduced isoproterenol-stimulated glycerol release by 10% in 3T3-L1 adipocytes, while its overexpression increased the glycerol release by 20%. Thus, TGH-2 may make a contribution to adipocyte lipolysis during period of increased energy demand. *Diabetes* 55: 2091–2097, 2006

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ATGL, adipose triglyceride lipase; BAT, brown adipose tissue; DMEM, Dulbecco's modified Eagle's medium; FFA, free fatty acid; HSL, hormone-sensitive lipase; iPLA2, Ca^{2+}-independent PLA2; PNPB, *p*-nitrophenyl butyrate; TGH, triacylglycerol hydrolase; WAT, white adipose tissue.

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Cellular accumulation of excessive neutral lipids underlies many diseases such as obesity, type 2 diabetes, and atherosclerosis, all of which are epidemic in industrialized countries. Therefore, elucidating the metabolic pathways that degrade excessive neutral lipids is of extreme importance in the prevention of the diseases caused by lipotoxicity (1).

Hormone-sensitive lipase (HSL) is an intracellular neutral lipase that catalyzes the hydrolysis of cellular triacylglycerol, diacylglycerol, monoacylglycerol, and cholesteryl ester, as well as other lipids (2,3). HSL is expressed in a wide variety of organs and cells including adipose tissues, heart, skeletal muscle, adrenal glands, testes, ovaries, and pancreatic β-cells and is under neural and hormonal control. To define the role of HSL in the hydrolysis of neutral lipids in vivo, several laboratories, including ours, have generated HSL-deficient ($HSL^{-/-}$) mice by targeted gene disruption (4–6). Although $HSL^{-/-}$ mice had a decreased ability to release free fatty acid (FFA) from adipocytes in response to the β-adrenergic stimulation both in vivo and in vitro, they were neither obese nor cold sensitive (4,5). The attenuated development of adipocyte-associated phenotypes may result from the presence of a residual triacylglycerol lipase activity in adipocytes that mediates lipolysis stimulated by both β-adrenergic agonist and tumor necrosis factor-α (7). These findings resulted in the use of a bioinformatic approach to search for a protein that shares several motifs with HSL. Of 33 genes that have not been annotated, only 1 shows hydrolytic activity toward triacylglycerol and is expressed in adipose tissue.

Here we report the structure, substrate specificity, subcellular localization, tissue distribution, and regulation of expression in adipose tissue of this enzyme, which we have designated as triacylglycerol hydrolase (TGH)-2 because of its striking sequence similarity to microsomal liver/adipocyte TGH originally reported by Lehner and colleagues (8,9).

RESEARCH DESIGN AND METHODS

Triolein, lecithin, BSA fraction V (BSA), leupeptin, pentadecanoic acid, *p*-nitrophenyl butyrate (PNPB), 2-monoolein, dioleoylglycerol, dioleoyl-phos-

phatidylserine, dioleoyl-phosphatidylcholine, dioleoyl-phosphatidic acid, dioleoyl-phosphatidylethanolamine, and phosphatidylinositol (18:0, 24:0) were purchased from Sigma. Dexamethasone, 3-isobutyl-2-methylxanthine, fatty acid–free BSA, isoproterenol, and sodium taurocholate were purchased from Wako Pure Chemicals (Osaka). Pioglitazone was provided by Takeda Pharmaceutical (Osaka). Tri[^{3}H]oleoylglycerol and cholesteryl [1-^{14}C]oleate were purchased from Applied Biosystems (Foster City, CA). Adenovirus plasmid carrying β-galactosidase (pAd-LacZ) has been described previously (10).

Database search and amino acid sequence analysis. We used the MOTIF search program in the GENES protein database of Kyoto Encyclopedia of Genes and Genomes (11) to search for proteins containing lipase consensus motifs and α/β hydrolase fold. We predicted the secondary structures of proteins with the programs PSIPRED and PHDsec and searched for their orthologue or paralogue of TGH-2 by SSDB in the Kyoto Encyclopedia of Genes and Genomes database (11). Alignment of the deduced protein sequence to other lipases, and calculation of the percent identity, were performed using the CLUSTAL W program.

Mice. C57BL/6 male mice were purchased from Clea (Tokyo, Japan). Mice were caged separately with 12-h light/dark cycles and given free access to standard chow diet containing 0.075% cholesterol (MF; Oriental Yeast, Osaka, Japan) in accordance with the regulations of the animal care committee of the University of Tokyo. For refeeding, animals were fasted for 24 h (from 2200 to 2200) and then refed for 12 h (2200–1000). For fasting, animals were fasted for 24 h (1000–1000).

Cell culture. HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal bovine serum. 3T3-L1 cells were cultured in medium A (DMEM containing 10% calf serum, supplemented with Ca-pantothenate and biotin). To induce differentiation of 3T3-L1 cells into mature adipocytes, 2-day postconfluent preadipocytes (day 0) were incubated with 1 μmol/l dexamethasone and 0.5 mmol/l 3-isobutyl-2-methylxanthine, 5 μg/ml insulin, and 1 μmol/l pioglitazone for 48 h, followed by treatment with 5 μg/ml insulin and 1 μmol/l pioglitazone for 48 h. After the incubation period, cells were switched to medium A, and the medium was renewed every other day.

Northern blot analyses. Ten micrograms of total RNA, which was isolated by TRIzol reagent (Invitrogen), were used for Northern blot analysis as described (12). Probes for murine TGH-1 (mouse orthologue of TGH) and TGH-2 were constructed from cDNA fragments amplified by RT-PCR using cDNA obtained from mouse liver as a template. Because TGH-1 and -2 are highly homologous proteins, the region that is specific to each mRNA and shares no sequence homology with the other was chosen for the construction of cDNA probe, i.e., from 1,130 to 1,260 bp for TGH-1 and from 1,130 to 1,260 bp for TGH-2. The cDNA fragment of HSL encoded by exon 8 was amplified by RT-PCR using cDNA obtained from mouse adipose tissue as a template and used as a probe (12).

Preparation of polyclonal antibody. To prepare polyclonal anti-mouse TGH-1 and -2 antisera, amino acid residues containing the catalytic domain of TGH-1 (amino acids 196–332) and TGH-2 (amino acids 196–333) were expressed in *Escherichia coli* as a glutathione S-transferase fusion protein, which was subsequently purified by glutathione affinity chromatography and used for immunization of rabbits according to the standard protocols (7). Cross-reactivities of the antisera against TGH-1 and -2 were tested using cell lysates overexpressing TGH-1 or -2 by adenovirus-mediated gene delivery.

Western blot analysis. Cells or tissues were homogenized in buffer A (50 mmol/l Tris-HCl, pH 7.0, 250 mmol/l sucrose, 1 mmol/l EDTA, and 2 μg/ml leupeptin) and centrifuged at 100,000*g* for 45 min at 4°C. The supernatant was used as S-100 cytosolic fraction, and the precipitates were resuspended and used as microsomal fraction (12). For Western blot analysis of fatcake proteins, white adipose tissue (WAT) was homogenized in buffer A and centrifuged at 100,000*g* for 45 min at 4°C, followed by extraction of fatcake proteins from the floating fat layer by the acetone precipitation method, essentially as described (7). Ten micrograms of protein from each fraction were subjected to Western blot analysis, as previously described, using an anti–TGH-1, anti–TGH-2, anti-HSL, or anti-perilipin antibody that was raised according to the described method (7). NIH Image (http://rsb.info.nih.gov/nih-image/) was used to compare the intensity of the bands.

Expression in mammalian cells. The expression vector was constructed by ligating the cDNA fragments, which were amplified by RT-PCR from mouse liver, into the TA cloning site of pTARGET vector (Promega). DNA transfection was performed with HEK293 cells plated on a six-well plate using SuperFect reagent (Qiagen). Expression vector (1.5 μg) was simultaneously transfected with pSV-βgal (0.5 μg) to measure transfection efficiency. The total amount of DNA in each transfection was adjusted to 1.5 μg/well with the mock vector. After a 48-h incubation, the cells were harvested and assayed for enzyme activity.

Construction of recombinant adenoviruses. Recombinant adenoviruses that carried murine TGH-1 and -2 cDNA under the control of cytomegalovirus promoter, designated as Ad-TGH-1 and Ad-TGH-2, respectively, were constructed as previously described (12). The recombinant adenoviruses were expanded in HEK293 cells and purified by cesium chloride ultracentrifugation (13). The purified viruses were stored in 10% (vol/vol) glycerol/PBS at −80°C. In our preparations, 1 m.o.i. (multiplicity of infection) corresponded to 25 particles of adenovirus per cell, and cells were infected at 1 m.o.i. for HEK293 cells and 1,000 m.o.i. for 3T3-L1 adipocytes. Seventy-two hours after the infection, the cells were used for the experiments.

RNA silencing. The following 21-mer sense strands of RNA oligonucleotides were designed as described (14): scrambled, 5′-CAGUCGCGUUUGCGACUG GdTdT-3′; HSL, 5′-GCCAAAGAUGAAGUGAGACdTdT-3′; TGH-1, 5′-AGAACA GCAGAGACUACCAGUdTdT-3′; and TGH-2, 5-GAAUGUAGUAGGAGACCAU dTdT-3′. The sense and antisense strands were synthesized, mixed, and annealed. At 5 days of differentiation, 3T3-L1 adipocytes were transfected with siRNA duplexes by electroporation as described (15). After incubation in medium A for 48 h, the cells were used for the experiments.

Enzyme assays. Cells were sonicated in buffer A and centrifuged at 15,000*g* for 10 min at 4°C. The supernatant was used for the enzyme assays. Neutral cholesteryl ester hydrolase or triacylglycerol lipase activities were measured using radiolabeled triolein or cholesterol oleate, respectively, as previously described (4). PNPB esterase activity was measured as previously described (16,17). Hydrolytic activities toward monoacylglycerol, diacylglycerol, and phospholipids were determined essentially as previously described (18,19). In brief, 2-monoolein, dioleoylglycerol, dioleoyl-phosphatidylserine, dioleoyl-phosphatidylcholine, dioleoyl-phosphatidic acid, and dioleoyl-phosphatidylethanolamine were incubated with cell extracts or tissue homogenates at 37°C for 1 h. Immediately thereafter, standard pentadecanoic acid (C15:0) was added to the reaction mixture and total lipids were extracted by Bligh and Dyer's method and reconstituted in chloroform/methanol (2:1 [vol/vol]). Mass spectrometry spectra were obtained by introducing the samples into a Quattro Micro tandem quadrupole mass spectrometer (Micromass, Manchester, U.K.) equipped with an ESI source at the flow rate of 30 μl/min. The eluting solvent used was acetonitrile/methanol/water (2:3:1 [vol/vol/vol]) containing 0.1% ammonium formate (pH 6.4). The mass spectrometer was operated in the negative ion scan mode. The nitrogen drying gas flow rate was 12 l/min and the temperature 80°C. The capillary voltage was set at 3.7 kV and cone voltage at 50 V. Amounts of oleic acid were deduced from its mass spectrometry spectra relative to that of standard pentadecanoic acid.

Lipolysis assay. The 3T3-L1 adipocytes either infected with the recombinant adenovirus or transfected with siRNA duplexes were cultured in 12-well plates. After incubation in medium A supplemented with 2% (wt/vol) fatty acid–free BSA in the absence of serum for 12 h, cells were treated with or without 10 mmol/l isoproterenol for the indicated amounts of time and the concentrations of glycerol and FFA in the media were determined by the Triglyceride-G kit and NEFA-C kit, respectively (Wako Pure Chemicals). Cellular protein contents were measured by bincinchoninic acid protein assay (Pierce) after solubulization in 0.1 N NaOH. Amounts of glycerol or FFA in the medium were normalized to cellular protein.

Statistical analyses. Results are presented as means ± SE. Student's *t* test was used to compare the means. All calculations were performed with StatView (version 5.0; SAS Institute) for Macintosh.

RESULTS

To search for enzymes with triacylglycerol lipase activity, we screened the gene database for murine and human proteins with structural homologies to known lipases, i.e., α/β hydrolase folds (20), the GXSXG active serine motif for serine esterases, and the His-Gly dipeptide motif that is present in 70–100 amino acids' NH_2-terminal of the catalytic site serine in many lipases (21), yielding 53 candidates that include HSL, lipoprotein lipase, hepatic lipase, endothelial lipase, pancreatic lipase, carboxyl ester lipase, gastric lipase, lysosomal acid lipase, monoacylglycerol lipase, and TGH. We selected 33 candidates that had not been previously annotated, and expressed them in HEK293 cells for measurement of triacylglycerol hydrolase activity. Northern blot analysis was used to verify the expression in mouse adipose tissue. Only one previously unannotated enzyme fulfilled these requirements, and we tentatively designated it as TGH-2 because it has sequence homology to TGH (22).

The murine gene for TGH-2 (National Center for Biotechnology Information nucleotide entry BC013479) en-

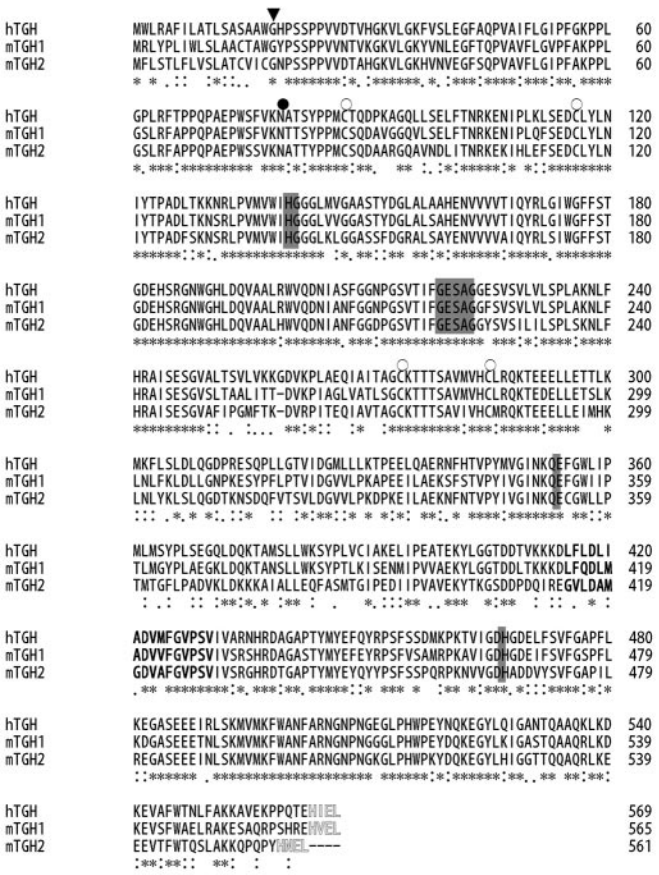

FIG. 1. Primary sequence of murine TGH-2 in comparison with human and murine TGH. The deduced amino acid sequence of murine TGH-1 (mTGH-1) and TGH-2 (mTGH-2) proteins are aligned and compared with human TGH (hTGH). The first Met residue is numbered as 1. The mature TGH proteins have been highly conserved in evolution, including *1*) conserved catalytic triad, Ser, Glu, His residues (shaded box); *2*) the His-Gly dipeptide motif (shaded box); *3*) the NH_2-glycosylation site (solid circle); and *4*) the signal peptide cleavage site (vertical arrow head), all of which are determined from the functional analysis of hTGH protein. Identical amino acids are shown by an asterisk (*). Amino acids considered strongly conserved are STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, and FYW and indicated by a colon (:). Amino acids considered weakly conserved are CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, and HFY and indicated by dots. The putative lipid binding domain is shown in boldface. Conserved cysteine residues that putatively form disulfide bonds are labeled with an open circle. A putative endoplasmic reticulum retrieval sequence (COOH-terminal HXEL) is indicated by outlined letters.

codes a 561–amino acid protein (NP_659179) with a calculated molecular mass of 61.6 kDa. Figure 1 shows alignments of amino acid sequences of murine TGH-2; its paralogue, TGH (22,23), which has also been designated as mouse carboxylesterase 3 and carboxylesterase MH1 (23); and its human orthologue (human carboxylesterase 1) (24,25). TGH-2 might be identical to carboxyesterase-ML1, based on the available partial amino acid sequence (26). TGH-2 shows 70.4% identity to murine TGH-1, which encodes a 565–amino acid protein. The human TGH gene encodes a 566–amino acid protein (NP_001257) with 77.9 and 67.0% identity to the murine TGH-1 and -2, respectively. The NH_2-terminal regions of 18 residues in both the murine and the human enzyme contain a predicted signal sequence that is cleaved in the mature human TGH protein (25). The Ser 221, Glu 353, and His 466 of the murine TGH-2 protein form a putative catalytic triad; this triad is highly conserved in human TGH, in which mutation of any of these triad residues to Ala abolishes TGH esterase activity (25). A potential NH_2-glycosylation site could be deduced at Asn 79 from the alignment with human TGH (25). The sequence contains four conserved Cys residues that are predicted to form disulfide bonds: Cys 87 to 116 and Cys 273 to Cys 284 (25). The protein sequence also contains a hydrophobic stretch of amino acids that may be involved in lipid binding: Gly 414 to Val 429. A COOH-terminal HXEL sequence possibly functions as an endoplasmic reticulum retrieval signal. In contrast to HSL, neither TGH-1 nor -2 contains putative phosphorylation site for protein kinase A.

A homology search revealed the presence of related proteins in a wide range of organisms, including animals, plants, and bacteria. In humans, two proteins, LOC390732 (XP_372639) and carboxylesterase 4–like (NP_057364), have a high degree of homology to human TGH (67.7 and 92.9%, respectively). These proteins appear to be distinct proteins and have been implicated as carboxylesterase. In mice, three other proteins, 2310039D24Rik (XP_134476), LOC244595 (XP_146438), and LOC382044 (XP_356117), have a high sequence similarity to murine TGH-2 (75.4, 67.5, and 63.8%, respectively). Homologous proteins are also found in *Drosophila melanogaster* and *Caenorhabditis elegans*. Interestingly, additional putative proteins sharing some degree of homology are present in *Arabidopsis thaliana*, *Cyanidioschyzon merolae*, and others in prokaryotes, including *Xanthomonas campestris*, *Bacillus subtilis*, and *Mycobacterium$_{avium}$ paratuberculosis*. The presence of TGH-1 and -2, as well as other related proteins, in such a wide variety of organisms suggests that TGH-1 and -2 comprise a subfamily of carboxyesterases mediating basic cellular functions that is not exclusive of higher organisms.

Northern blot analysis was performed to compare the mRNA expression of TGH-2 with that of HSL and TGH-1 in various tissues. The tissue distribution of TGH-2 mRNA expression is remarkably similar to that of TGH-1 except in intestine: they are expressed at high levels in WAT, brown adipose tissue (BAT), liver, and kidney but to a lesser degree in adrenal, ovary, and heart (Fig. 2). TGH-2, but not TGH-1, is highly expressed in intestine. To determine whether they are expressed in adipocytes in a differentiation-dependent manner, we performed Northern blot analysis of TGH-1, TGH-2, and HSL at 0, 2, 5, and 8 days postinduction of 3T3-L1 adipocyte differentiation (Fig. 2*B*). Like HSL, mRNA expression of both TGH-1 and -2 was induced during differentiation of 3T3-L1 adipocytes.

Because of the high sequence similarities between the peptides that were used for immunization, it is possible that the antibodies cross-react with TGH-1 and -2. To exclude this possibility, we performed Western blot analysis using the antibodies against TGH-1 or -2 (Fig. 3*A*). The antibody against TGH-1 detected TGH-1 but not TGH-2, which were both overexpressed in HEK293 cells over the background level. On the other hand, the antibody against TGH-2 detected TGH-2 but not TGH-1, which were both overexpressed in HEK293 cells over the background level. These results indicate that cross-reaction of the antibodies is negligible.

To determine subcellular localization of TGH-1, TGH-2, and HSL, we performed subcellular fractionation of WAT and BAT by ultracentrifugation. Substantial amounts of TGH-1, TGH-2, and HSL were present in fatcake fraction (Fig. 3*B*), corroborating the idea that TGH-1 and -2 mediate hydrolysis of neutral lipids in fat droplets where perilipin colocalizes. Unlike fatcake-specific perilipin, TGH-1, TGH-2, and HSL were also present in cytosolic and

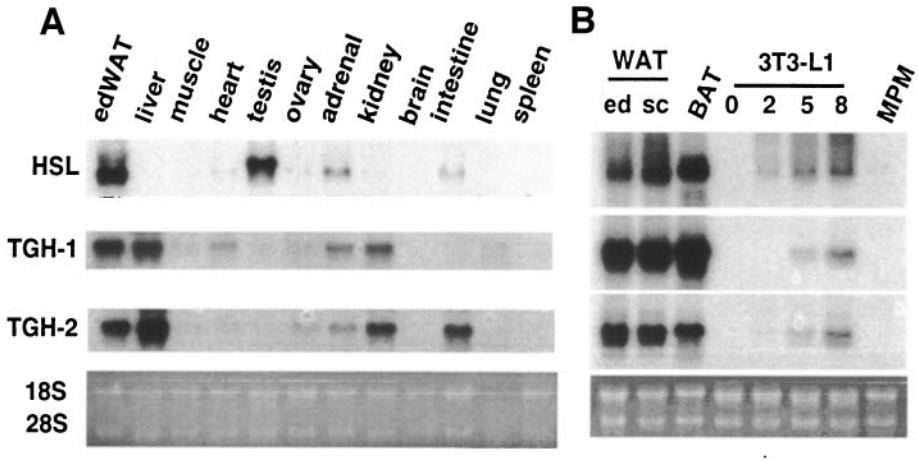

FIG. 2. Northern blot analysis of HSL, TGH-1, and TGH-2. Total RNA (10 μg) from tissues and mouse peritoneal macrophages (MPM) of C57BL/6J mice (*A* and *B*) and 3T3-L1 adipocytes at various stages of differentiation (*B*) were subjected to Northern blot analysis. Specific mRNAs were detected with a radiolabeled cDNA probes for HSL, TGH-1, or TGH-2. Ethidium bromide staining of gels is shown as a loading control. ed, epididymal; sc, subcutaneous.

microsomal fraction. There were no obvious differences in their distribution between subcutaneous and epididymal WAT and BAT (Fig. 3*C*).

To determine the types of lipids that TGH-2 hydrolyzes, we infected HEK293 cells with recombinant adenoviruses carrying expression cassettes of LacZ, HSL, TGH-1, or TGH-2. Whole-cell extracts of cells infected with Ad-TGH-1 or Ad-TGH-2 exhibited substantial amounts of hydrolytic activities toward PNPB, triacylglycerol, or monoacylglycerol (Table 1). Both TGH-1 and -2 hydrolyzed PNPB, a short-chain fatty acid ester, more preferentially than triolein, a long-chain fatty acid ester. Their preference was ~50- to 60-fold higher than that of HSL. However, neither TGH-1 nor TGH-2 showed detectable hydrolytic activities toward diacylglycerol, cholesteryl ester, or phospholipids. Thus, TGH-2 is indistinguishable from TGH-1 in their substrate specificity. The substrate specificity of HSL is different from TGH-1 and -2 in that it prefers triacylglycerol, diacylglycerol, and cholesteryl ester compared with TGH-1 and -2.

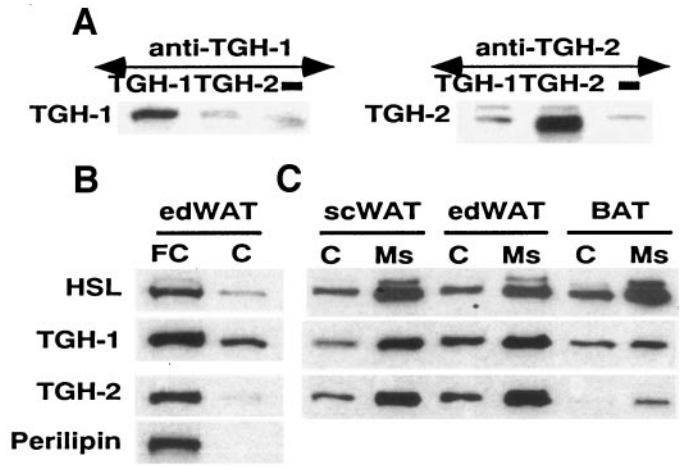

FIG. 3. Western blot analysis of HSL, TGH-1, and TGH-2. *A*: Cross-reaction of the antibodies. We overexpressed TGH-1 or -2 in HEK293 cells by plasmid transfection and used these cell lysate for Western blot analysis. Anti–TGH-1 and anti–TGH-2 detected the overexpressed TGH-1 and -2, respectively, but did not cross-react with TGH-2 and -1 over the background levels, respectively. *B* and *C*: Subcellular distribution of the lipases. Tissues of C57BL/6J mice were homogenized and centrifuged at 100,000*g*. Fatcake proteins (FC), which were extracted from floating fat layers, and supernatant (C) were subjected to Western blot analysis (*B*). Supernatant or microsomal (Ms) fractions were subjected to Western blot analysis (*C*). ed, epididymal; sc, subcutaneous.

To determine whether TGH-2 is a lipolytic enzyme that contributes to the lipolysis in adipocytes, we examined the effects of knockdown of TGH-2 by RNA silencing on the lipolysis in 3T3-L1 adiocytes (Fig. 4). Western blot analyses revealed that transfection of siRNA duplexes for HSL, TGH-1, or TGH-2 inhibited the expression of respective lipases by 82, 86, and 86%, respectively (Fig. 4*A*). When the cells were not stimulated with isoproterenol, the RNA interference for HSL, TGH-1, or TGH-2 did not significantly inhibit the amounts of either glycerol or FFA release at 3 h of the incubation ($n = 3$). When the cells were stimulated by isoproterenol, the RNA interference for HSL, TGH-1, or TGH-2 inhibited the amounts of glycerol release by 30% ($P < 0.05$), −2%, and 10% ($P < 0.05$), respectively (Fig. 4*B*), whereas it inhibited the amounts of FFA release by 36% ($P < 0.05$), 3%, and 6%, respectively, at 3 h after the stimulation with isoproterenol ($n = 3$).

To determine whether TGH-2 is involved in lipolysis in adipocytes, we infected 3T3-L1 adipocytes with Ad-TGH-2 and measured the amounts of FFA and glycerol released with or without isoproterenol (Fig. 5). In the absence of isoproterenol, we could not detect any differences in lipolysis. Isoproterenol stimulated basal lipolysis, as shown in the cells infected with Ad-LacZ. Overexpression of TGH-2 resulted in 3.6- and 2.0-fold increases in the protein expression in cytosolic and microsomal fractions, respectively. These changes were associated with further increases in the release of glycerol by 20% ($P < 0.05$) (Fig. 5*B*) and FFA by 26% (Fig. 5*C*) at 3 h after the stimulation with isoproterenol, supporting the idea that TGH-2 is involved in lipolysis.

To explore the possibility that mRNA expression of TGH-1 and -2 is regulated in response to feeding cycle, we examined changes in the mRNA expression of TGH-1 and -2 in various tissues, including WAT and BAT, after fasting or refeeding in comparison with the expression of HSL (Fig. 6). In contrast to HSL, whose expression was relatively stable during the fasting/refeeding cycle, mRNA levels of both TGH-1 and -2 were relatively low in a refed state and increased by fasting in WAT and BAT. In the liver, intestine, and kidney, TGH-2 was upregulated in the refed state, whereas this did not occur in WAT.

DISCUSSION

Here we report identification and characterization of a previously undescribed TGH that is predominantly expressed in adipose tissue. Because this enzyme has high

TABLE 1
Comparison of substrate specificity of HSL, TGH-1, and TGH-2

Substrates	LacZ	HSL	TGH-1	TGH-2
PNPB (nmol · min^{-1} · mg^{-1})	59	5,258	9,494 ± 1,268	14,349 ± 2,289
Triolein (pmol · min^{-1} · mg^{-1})	78 ± 20	9,581	429 ± 15	506 ± 35
Diolein (pmol · min^{-1} · mg^{-1})	10.3	113.3	10.5	10.0
Monoolein (pmol · min^{-1} · mg^{-1})	40.5	184.5	190.0	217.7
Cholesteryl oleate (pmol · min^{-1} · mg^{-1})	8.3 ± 4.5	1,184 ± 22	3.7 ± 0.5	2.6 ± 0.3
Phospholipid (pmol · min^{-1} · mg^{-1})	7.3	15.2	9.0	9.0

Data are means (duplicate measurements) or means ± SE (triplicate measurements). We overexpressed LacZ, HSL, TGH-1, or TGH-2 by adenovirus-mediated gene delivery and used whole-cell extracts for measurements of hydrolytic activities. Hydrolytic activities for PNPB were measured by colorimetric assay, hydrolytic activities for triolein and cholesteryl oleate were measured by determining the release of radiolabeled oleate, and hydrolytic activities for monoolein, diolein, and phospholipids were measured by determining the release of oleic acid by mass spectrometry. Since different buffers were used for different assays, care should be taken when making comparison between different methods.

sequence homology to TGH, we have designated the original TGH as TGH-1 and the newly identified one as TGH-2. These enzymes belong to a carboxylesterase family and have remarkable similarity in terms of tissue distribution, subcellular localization, substrate specificity, and regulation.

Recently, three laboratories have independently reported the cloning of a novel triacylglycerol lipase expressed in adipocytes (27–29). This protein, designated as desnutrin and adipose triglyceride lipase (ATGL) in mice and Ca^{2+}-independent PLA2 (iPLA2)ζ in humans according to each laboratory, contains α/β hydrolase fold and GXSXG active serine motif, is expressed in adipose tissue, is localized in both cytosol and lipid droplets, and mediates hydrolysis of triacylglycerol but not cholesteryl ester or diacylglycerol, thereby contributing to adipocyte lipolysis (28) and acylglycerol transacylation to synthesize diacylglycerol or triacylglycerol (29). The *Drosophila* orthologue Brummer lipase has also been reported (30). There are two other paralogues of ATGL: adiponutrin in mice (31), which is also called iPLA2ε in humans (29), and iPLA2η in humans (29). Desnutrin/ATGL/iPLA2ζ was not found in our database search because it does not contain an oxyanion His-Gly dipeptide motif. Instead, it contains a patatin domain that is conserved from plants to mammals (28). According to Zimmermann et al. (28) and Kershaw et al. (32), silencing ATGL gene by siRNA or antisense RNA markedly decreased lipolysis in stimulated and nonstimulated 3T3-L1 adipocytes, supporting the notion that desnutrin/ATGL/iPLA2ζ is the major non-HSL lipase mediating lipolysis in adipocytes.

Despite these substantial evidences for the involvement of desnutrin/ATGL/iPLA2ζ in the lipolysis, TGH-2 as well as TGH-1 may contribute to the residual triacylglycerol lipase activity in HSL-deficient adipocytes (4,6,7). First, the substrate specificity of TGH-1 and -2 is consistent with the characteristics of the residual lipase activity in HSL-deficient WAT: substantial triacylglycerol lipase activity with almost complete absence of neutral cholesteryl ester hydrolase and diacylglycerol lipase activity. When overexpressed in HEK293 cells, TGH-1 and -2 were both able to hydrolyze triacylglycerol and monoacylglycerol but not diacylglycerol, cholesteryl ester, or phospholipids (Table 1). Second, expression of TGH-1 and -2 was increased during the differentiation of 3T3-L1 adipocytes (Fig. 2*B*). Third, both fatcake and cytosolic fractions contain TGH-1 and -2 , which is consistent with the residual triacylglycerol lipase activity present in HSL-deficient WAT (Fig. 3) (7). Indeed, Soni et al. (17) have recently reported that TGH, carboxylesterase 3, is the major protein present in the second peak, with substantial PNPB esterase activity that was eluted from oleic acid–agarose chromatography of infranatant as well as fatcake fractions from mouse adipose tissue. However, our studies on the inhibition or overexpression of TGH-1 and -2 (Figs. 4 and 5) indicate that TGH-1 and -2 appear to make a relatively minor contribution to net triacylglycerol hydrolysis in adipose tissues compared with other adipocyte lipases such as desnutrin/ATGL/iPLA2ζ and HSL.

The robust induction of the expression of TGH-1 and -2 during fasting is intriguing from the point of view of energy homeostasis. Fasting also induces the expression of desnutrin/ATGL/iPLA2ζ (27,32). Induction of these multiple lipases is reasonable physiological response to fasting/starvation if these enzymes mediate lipolysis that may have evolved for adaptation to fasting/starvation. Desnu-

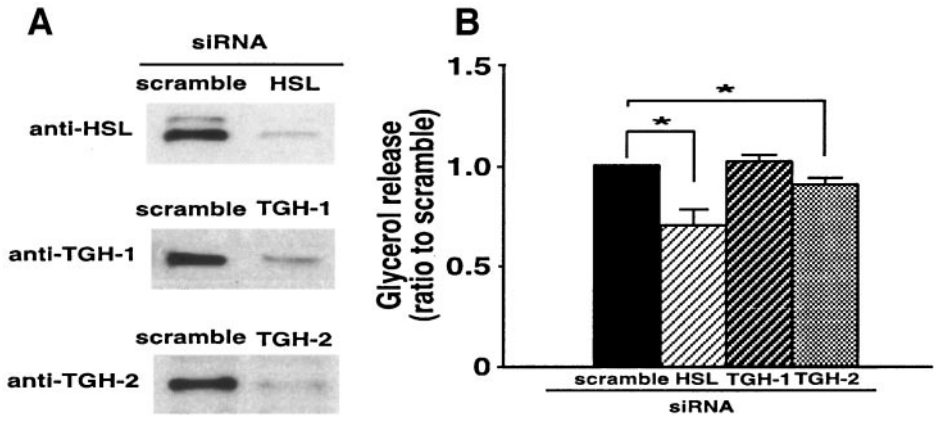

FIG. 4. Inhibition of TGH-2 by RNA silencing and its effect on lipolysis in 3T3-L1 adipocytes. At 5 days of differentiation, 3T3-L1 adipocytes were transfected with siRNA duplexes by electroporation. After 48 h, the cells were treated with isoproterenol for 3 h. Cell lysates were used for Western blot analysis (*A*), and the amounts of glycerol in the medium were measured and divided by the values for scramble siRNA in each experiment after normalizing to the cellular protein (*B*). Data are expressed as means ± SE of four independent experiments. **P* < 0.05 vs. control scramble siRNA.

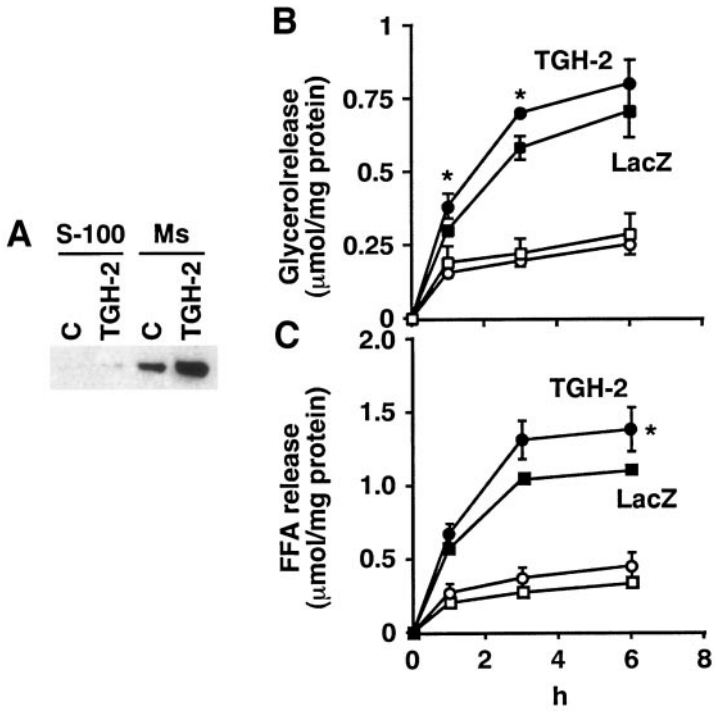

FIG. 5. Overexpression of TGH-2 by recombinant adenovirus and its effect on lipolysis in 3T3-L1 adipocytes. Recombinant adenoviruses carrying LacZ (Ad-LacZ) or TGH-2 (Ad-TGH2) were used to infect 3T3-L1 adipocytes on day 8 of differentiation, and experiments were performed 3 days after infection. *A*: Overexpression of TGH-2 protein was verified by Western blot analysis using cytosolic (S-100) and microsomal (Ms) fraction of 3T3-L1 adipocytes infected with Ad-TGH2 or Ad-LacZ for 72 h. *B* and *C*: Cells overexpressing TGH-2 (circle) or LacZ (square) were incubated in DMEM/2% fatty acid–free BSA in the absence (□ and ○) or presence (■ and ●) of 10 μmol/l isoproterenol for the indicated times, and glycerol (*B*) and FFA (*C*) released into the media were measured. Data are expressed as means ± SE of triplicate measurements. *$P < 0.05$, vs. control Ad-LacZ–infected cells. C, control; Ms, microsomal fraction.

trin/ATGL/iPLA2ζ is primarily localized in cytosol and fatcake (28), while TGH-1 and -2 are mainly localized in fatcake and microsome (Fig. 3*B* and *C*). This difference in subcellular localization suggests that these lipases play distinct roles in response to fasting.

In addition to adipose tissues, TGH-1 and -2 are expressed in a wide variety of nonadipose tissues. Liver is the organ where both TGH-1 and -2 are most abundantly expressed. Lehner and colleagues (9,33,34) have previously assigned the function of hepatic TGH to VLDL assembly. This may be the case with TGH-2, since the expression of TGH-2 was increased in a refed state after VLDL production was stimulated. A similar role in lipoprotein assembly can be assigned to TGH-2, which is uniquely expressed in intestine where chylomicron is produced. Clearly, these intriguing possibilities await further investigation.

It is also noteworthy that TGH-1 and -2 have a much higher substrate specificity for short-chain carbonic acid esters than long-chain triacylglycerol (Table 1). Indeed, murine TGH is identical to carboxylesterase 3, also designated carboxylesterase MH1, which has been identified as a carboxylesterase isozyme induced by di-(2-ethylhexyl)phthalate, a peroxisome proliferator (23). Furthermore, based on the available partial amino acid sequence, TGH-2 itself might be identical to carboxylesterase-ML1 (26,35). From these considerations, both enzymes might be better designated by their original names, carboxylesterase-3 for TGH-1 and carboxylesterase-ML1 for TGH-2.

Our results in Table 1 do not appear to be consistent with the previous findings that HSL hydrolyzes diacylglycerol better than triacylglycerol (36,37). These contradictions might result from the difference in the sensitivity of two different methods used in our study: measurements of the end products, FFAs, by radioactivity versus mass spectrometry. The method that used mass spectrometry omitted phospholipids from the reaction mixture to avoid generation of FFAs from the hydrolysis of phospholipids; this could profoundly impair the sensitivity of the assay. When the triacylglycerol lipase activities of HSL were directly measured by the method that used mass spectrometry, its specific triacylglycerol lipase activity was calculated to be 6.9 pmol · min^{-1} · mg^{-1}. This value was 15-fold smaller than the diacylglycerol lipase activity mea-

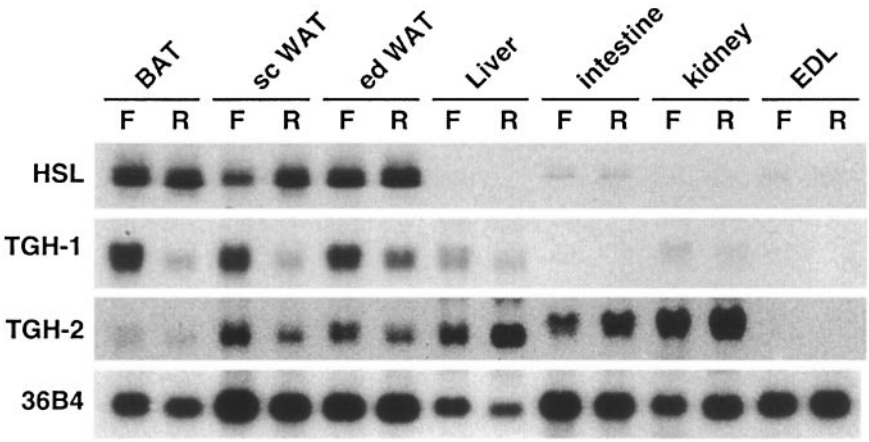

FIG. 6. Changes in the expressions of TGH-1 and -2 mRNA during fasting/refeeding. Total RNA (10 μg) from tissues of mice fasted for 24 h (F) or fasted and then refed for 12 h (R) were subjected to Northern blot analysis. Specific mRNAs were detected with a radiolabeled cDNA probes for HSL, TGH-1, TGH-2, or 36B4. ed, epididymal; EDL, extensor digitorum longus; sc, subcutaneous.

sured by the same method (103 pmol · min^{-1} · mg^{-1}) (Table 1), roughly corroborating the ratios in the literature (36,37).

In conclusion, we have identified a novel member of the carboxylesterase family, TGH-2, with structural, catalytic, and expressional similarity to TGH or TGH-1. TGH-1 and -2 are highly expressed in adipocytes and are transcriptionally regulated in response to changes in nutritional conditions, suggesting their potential role in adipocyte lipolysis. The elucidation of the true function of these "redundant" lipases awaits further in vivo studies of the gain or loss of function by pharmacologic and/or genetic manipulation.

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REFERENCES

1. Unger RH: Lipotoxic diseases. *Annu Rev Med* 53:319–336, 2002
2. Holm C, Osterlund T, Laurell H, Contreras JA: Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Annu Rev Nutr* 20:365–393, 2000
3. Kraemer FB, Shen WJ: Hormone-sensitive lipase: control of intracellular tri-(di-)acylglycerol and cholesteryl ester hydrolysis. *J Lipid Res* 43:1585–1594, 2002
4. Osuga J, Ishibashi S, Oka T, Yagyu H, Tozawa R, Fujimoto A, Shionoiri F, Yahagi N, Kraemer FB, Tsutsumi O, Yamada N: Targeted disruption of hormone-sensitive lipase results in male sterility and adipocyte hypertrophy, but not in obesity. *Proc Natl Acad Sci U S A* 97:787–792, 2000
5. Wang SP, Laurin N, Himms-Hagen J, Rudnicki MA, Levy E, Robert MF, Pan L, Oligny L, Mitchell GA: The adipose tissue phenotype of hormone-sensitive lipase deficiency in mice. *Obes Res* 9:119–128, 2001
6. Haemmerle G, Zimmermann R, Hayn M, Theussl C, Waeg G, Wagner E, Sattler W, Magin TM, Wagner EF, Zechner R: Hormone-sensitive lipase deficiency in mice causes diglyceride accumulation in adipose tissue, muscle, and testis. *J Biol Chem* 277:4806–4815, 2002
7. Okazaki H, Osuga J, Tamura Y, Yahagi N, Tomita S, Shionoiri F, Iizuka Y, Ohashi K, Harada K, Kimura S, Gotoda T, Shimano H, Yamada N, Ishibashi S: Lipolysis in the absence of hormone-sensitive lipase: evidence for a common mechanism regulating distinct lipases. *Diabetes* 51:3368–3375, 2002
8. Lehner R, Verger R: Purification and characterization of a porcine liver microsomal triacylglycerol hydrolase. *Biochemistry* 36:1861–1868, 1997
9. Lehner R, Vance DE: Cloning and expression of a cDNA encoding a hepatic microsomal lipase that mobilizes stored triacylglycerol. *Biochem J* 343:1–10, 1999
10. Ye X, Robinson MB, Batshaw ML, Furth EE, Smith I, Wilson JM: Prolonged metabolic correction in adult ornithine transcarbamylase-deficient mice with adenoviral vectors. *J Biol Chem* 271:3639–3646, 1996
11. Kanehisa M, Goto S, Kawashima S, Nakaya A: The KEGG databases at GenomeNet. *Nucleic Acids Res* 30:42–46, 2002
12. Okazaki H, Osuga J, Tsukamoto K, Isoo N, Kitamine T, Tamura Y, Tomita S, Sekiya M, Yahagi N, Iizuka Y, Ohashi K, Harada K, Gotoda T, Shimano H, Kimura S, Nagai R, Yamada N, Ishibashi S: Elimination of cholesterol ester from macrophage foam cells by adenovirus-mediated gene transfer of hormone-sensitive lipase. *J Biol Chem* 277:31893–31899, 2002
13. Tsukamoto K, Smith P, Glick JM, Rader DJ: Liver-directed gene transfer and prolonged expression of three major human ApoE isoforms in ApoE-deficient mice. *J Clin Invest* 100:107–114, 1997
14. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T: Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411:494–498, 2001
15. Jiang ZY, Zhou QL, Coleman KA, Chouinard M, Boese Q, Czech MP: Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. *Proc Natl Acad Sci U S A* 100:7569–7574, 2003
16. Shirai K, Jackson RL: Lipoprotein lipase-catalyzed hydrolysis of p-nitrophenyl butyrate: interfacial activation by phospholipid vesicles. *J Biol Chem* 257:1253–1258, 1982
17. Soni KG, Lehner R, Metalnikov P, O'Donnell P, Semache M, Gao W, Ashman K, Pshezhetsky AV, Mitchell GA: Carboxylesterase 3 (EC 3.1.1.1) is a major adipocyte lipase. *J Biol Chem* 279:40683–40689, 2004
18. Hosono H, Aoki J, Nagai Y, Bandoh K, Ishida M, Taguchi R, Arai H, Inoue K: Phosphatidylserine-specific phospholipase A1 stimulates histamine release from rat peritoneal mast cells through production of 2-acyl-1-lysophosphatidylserine. *J Biol Chem* 276:29664–29670, 2001
19. Hiramatsu T, Sonoda H, Takanezawa Y, Morikawa R, Ishida M, Kasahara K, Sanai Y, Taguchi R, Aoki J, Arai H: Biochemical and molecular characterization of two phosphatidic acid-selective phospholipase A1s, mPA-PLA1alpha and mPA-PLA1beta. *J Biol Chem* 278:49438–49447, 2003
20. Ollis DL, Cheah E, Cygler M, Dijkstra B, Frolow F, Franken SM, Harel M, Remington SJ, Silman I, Schrag J, Sussman JL, Verschueren KHG, Goldman A: The alpha/beta hydrolase fold. *Protein Eng* 5:197–211, 1992
21. Feller G, Thiry M, Gerday C: Nucleotide sequence of the lipase gene lip2 from the antarctic psychrotroph Moraxella TA144 and site-specific mutagenesis of the conserved serine and histidine residues. *DNA Cell Biol* 10:381–388, 1991
22. Dolinsky VW, Sipione S, Lehner R, Vance DE: The cloning and expression of a murine triacylglycerol hydrolase cDNA and the structure of its corresponding gene. *Biochim Biophys Acta* 1532:162–172, 2001
23. Furihata T, Hosokawa M, Koyano N, Nakamura T, Satoh T, Chiba K: Identification of di-(2-ethylhexyl) phthalate-induced carboxylesterase 1 in C57BL/6 mouse liver microsomes: purification, cDNA cloning, and baculovirus-mediated expression. *Drug Metab Dispos* 32:1170–1177, 2004
24. Alam M, Ho S, Vance DE, Lehner R: Heterologous expression, purification, and characterization of human triacylglycerol hydrolase. *Protein Expr Purif* 24:33–42, 2002
25. Alam M, Vance DE, Lehner R: Structure-function analysis of human triacylglycerol hydrolase by site-directed mutagenesis: identification of the catalytic triad and a glycosylation site. *Biochemistry* 41:6679–6687, 2002
26. Satoh T, Hosokawa M: The mammalian carboxylesterases: from molecules to functions. *Annu Rev Pharmacol Toxicol* 38:257–288, 1998
27. Villena JA, Roy S, Sarkadi-Nagy E, Kim KH, Sul HS: Desnutrin, an adipocyte gene encoding a novel patatin domain-containing protein, is induced by fasting and glucocorticoids: ectopic expression of desnutrin increases triglyceride hydrolysis. *J Biol Chem* 279:47066–47075, 2004
28. Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M, Lass A, Neuberger G, Eisenhaber F, Hermetter A, Zechner R: Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science* 306:1383–1386, 2004
29. Jenkins CM, Mancuso DJ, Yan W, Sims HF, Gibson B, Gross RW: Identification, cloning, expression, and purification of three novel human calcium-independent phospholipase A2 family members possessing triacylglycerol lipase and acylglycerol transacylase activities. *J Biol Chem* 279:48968–48975, 2004
30. Gronke S, Mildner A, Fellert S, Tennagels N, Petry S, Muller G, Jackle H, Kuhnlein RP: Brummer lipase is an evolutionary conserved fat storage regulator in Drosophila. *Cell Metab* 1:323–330, 2005
31. Baulande S, Lasnier F, Lucas M, Pairault J: Adiponutrin, a transmembrane protein corresponding to a novel dietary- and obesity-linked mRNA specifically expressed in the adipose lineage. *J Biol Chem* 276:33336–33344, 2001
32. Kershaw EE, Hamm JK, Verhagen LA, Peroni O, Katic M, Flier JS: Adipose triglyceride lipase: function, regulation by insulin, and comparison with adiponutrin. *Diabetes* 55:148–157, 2006
33. Gilham D, Ho S, Rasouli M, Martres P, Vance DE, Lehner R: Inhibitors of hepatic microsomal triacylglycerol hydrolase decrease very low density lipoprotein secretion. *FASEB J* 17:1685–1687, 2003
34. Gilham D, Alam M, Gao W, Vance DE, Lehner R: Triacylglycerol hydrolase is localized to the endoplasmic reticulum by an unusual retrieval sequence where it participates in VLDL assembly without utilizing VLDL lipids as substrates. *Mol Biol Cell* 16:984–996, 2005
35. Satoh T, Hosokawa M: Molecular aspects of carboxylesterase isoforms in comparison with other esterases. *Toxicol Lett 82-* 83:439–445, 1995
36. Fredrikson G, Stralfors P, Nilsson NO, Belfrage P: Hormone-sensitive lipase of rat adipose tissue. Purification and some properties. *J Biol Chem* 256:6311–6320, 1981
37. Ben Ali Y, Chahinian H, Petry S, Muller G, Carriere F, Verger R, Abousalham A: Might the kinetic behavior of hormone-sensitive lipase reflect the absence of the lid domain? *Biochemistry* 43:9298–9306, 2004