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Carboxylesterase 3 (EC 3.1.1.1) Is a Major Adipocyte Lipase*

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Hydrolysis of triglycerides is central to energy homeostasis in white adipose tissue (WAT). Hormone-sensitive lipase (HSL) was previously felt to mediate all lipolysis in WAT. Surprisingly, HSL-deficient mice show active HSL-independent lipolysis, suggesting that other lipase(s) also mediate triglyceride hydrolysis. To clarify this, we used functional proteomics to detect non-HSL lipase(s) in mouse WAT. After cell fractionation of intra-abdominal WAT, most non-HSL neutral lipase activity is localized in the 100,000 × *g* infranatant and fat cake fractions. By oleic acid-linked agarose chromatography of infranatant followed by elution in a 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid gradient, we identified two peaks of esterase activity using *p*-nitrophenyl butyrate as a substrate. One of the peaks contained most of the lipase activity. In the corresponding fractions, gel permeation chromatography and SDS-PAGE, followed by tandem mass spectrometric analysis of excised Coomassie Blue-stained peptides, revealed carboxylesterase 3 (triacylglycerol hydrolase (TGH); EC 3.1.1.1). TGH is also the principle lipase of WAT fat cake extracts. Partially purified WAT TGH had lipase activity as well as lesser but detectable neutral cholesteryl ester hydrolase activity. Western blotting of subcellular fractions of WAT and confocal microscopy of fibroblasts following *in vitro* adipocytic differentiation are consistent with a distribution of TGH to endoplasmic reticulum, cytosol, and the lipid droplet. TGH is responsible for a major part of non-HSL lipase activity in WAT *in vitro* and may mediate some or all HSL-independent lipolysis in adipocytes.

Lipolysis in white adipose tissue (WAT)[1] exerts a major influence on circulating free fatty acid levels and is an important determinant of energy homeostasis and of WAT mass. In WAT adipocytes, hormone-sensitive lipase (HSL) (EC 3.1.1.3) is an 84-kDa cytoplasmic protein that can cleave fatty acids from the first and third carbons of triacylglycerol (1) and can also hydrolyze cholesteryl esters (2–4). Until recently, HSL was considered to be the principle or the only mediator of lipolysis in WAT. However, gene-targeted HSL-deficient mice with no detectable HSL show active HSL-independent lipolysis (5, 6). Although HSL −/− mice have a severely blunted response to adrenergic stimulation (6), the basal lipolytic rate of isolated adipocytes was somewhat greater in HSL −/− cells than in normal cells (5, 6). In HSL −/− mouse embryonic fibroblasts, the basal lipolytic rate is equal in HSL −/− cells and controls. In homogenates of WAT (5) and of embryonic fibroblasts from HSL −/− mice (7), total TAG hydrolase (lipase) activity is 40−45% of that of normal mice.

Therefore, HSL appears to be responsible for hormone-stimulated lipolysis, but the enzyme(s) mediating basal lipolysis are unknown. We thus attempted to identify non-HSL lipase(s) that may be responsible for HSL-independent lipolysis in WAT. We report that carboxylesterase 3 (triacylglycerol hydrolase; TGH), a protein of the endoplasmic reticulum lumen previously implicated in hepatic very low density lipoprotein synthesis (8), accounts for a major fraction of non-HSL TAG hydrolase activity in WAT.

EXPERIMENTAL PROCEDURES

Chemicals—Oleic acid, triolein, phosphatidylcholine, phosphatidylinositol, fatty acid-free bovine serum albumin, *p*-nitrophenyl butyrate, CHAPS, and the protease inhibitors antipain, pepstatin, and leupeptin were purchased from Sigma. Affi-Gel 102 (aminoalkyl agarose) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were purchased from Bio-Rad. Glycerol tri-[9,10-^{3}H]oleate and cholesteryl [1-^{14}C]oleate were from Amersham Biosciences. ECL Western blotting reagents were from Roche Applied Science. Dulbecco's modified Eagle's medium and fetal bovine serum were from Invitrogen.

Antibodies—Anti-α-glucosidase II and anti-calnexin monoclonal antibodies were from Stressgen Biotechnologies (Victoria, Canada). Polyclonal guinea pig anti-perilipin antibody was from Research Diagnostics Inc. (Flanders, NJ). Alexa Fluor 488 donkey anti-rabbit IgG, Alexa Fluor 594 goat anti-guinea pig IgG, and ProLong Antifade kit were from Molecular Probes Inc. (Eugene, OR).

Mice—For WAT protein isolation, C57BL/6 mice were obtained from Charles River (Montreal, Canada). For comparison between HSL +/+ and HSL −/− mice, we used gene-targeted HSL-deficient mice (6) and control littermates. Ages were as indicated throughout.

Enzyme Assays—The activities of esterase (PNPB hydrolase), lipase (glycerol trioleate hydrolase), and neutral cholesteryl oleate hydrolase (NCEH) were assayed as described (9).

Briefly, to assay PNPB esterase, 34.8 μl of PNPB were dissolved in 1 ml of acetonitrile. For each assay, 10 μl of PNPB/acetonitrile were added to 990 μl of buffer (0.1 M phosphate and 150 mM NaCl, pH 7.5). Following the addition of 50 μl of sample, tubes were incubated at 37 °C for 10 min. The reaction was terminated by adding 3.25 ml of methanol/chloroform/heptane (10:9:7), vigorously vortexed for 10 s, and then

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[1] The abbreviations used are: WAT, white adipose tissue; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; MS, mass spectrometry; NCEH, neutral cholesteryl ester hydrolase; PNPB, *p*-nitrophenyl butyrate; TAG, triglyceride; TGH, triacylglycerol hydrolase.

TABLE I
Neutral glycerol trioleate hydrolase (top) and NCEH (bottom) activities in different subcellular fractions of adipocytes

Fractions	Lipase activity		
	Total	Percentage	Specific
	pmol/min	*%*	*pmol/min/mg of protein*
Homogenate	5442	100	134.98
Nuclear	104	1.9	44
Heavy mitochondrial	55	1	44
Light mitochondrial	78	1.4	38
Microsomal	240	4.4	71
Cytosol	3786	70	118
Fat cake	487	8.9	685
	NCEH activity		
	Total	Percentage	Specific
	pmol/min	*%*	*pmol/min/mg of protein*
Homogenate	3345	100	83
Nuclear	81	2.4	34
Heavy mitochondrial	49	1.46	40
Light mitochondrial	194	5.8	94
Microsomal	460	13.8	136
Cytosol	2216	66.2	69
Fat cake	310	9.2	87

centrifuged at 800 × g for 20 min at 21 °C. The upper phase was collected and afterward subjected to incubation at 42 °C for 3 min. The absorbance of released *p*-nitrophenol was measured at 400 nm. *p*-Nitrophenol concentration was calculated using its extinction coefficient of 12,000.

For lipase activity, in a 4-ml glass vial, 5.9 mg of triolein, including 50×10^6 cpm of [^{3}H]triolein in toluene, plus a phospholipid mixture (30 μl, 20 mg/ml phosphatidylcholine/phosphatidylinositol, 3:1) were dissolved in chloroform. Solvents were evaporated under a gentle stream of N_2. 2 ml of 0.1 M potassium phosphate, pH 7.5, was added and sonicated twice in an ice bath each for 1 min, separated by a 1-min interval. An additional 1.0 ml of 0.1 M potassium phosphate, pH 7.5, was added, and the mixture was sonicated 4 × 30 s on ice with a 30-s interval between sonications. Then 1.0 ml of 20% fatty acid-free bovine serum albumin (BSA) prepared in 0.1 M potassium phosphate (pH 7.5) was added. For each assay, 100 μl of substrate was mixed with 100 μl of sample and incubated for 30 min at 37 °C without shaking. The reaction was terminated by the addition of 3.25 ml methanol/chloroform/heptane (10:9:7). 1.05 ml of 0.1 M potassium carbonate, 0.1 M boric acid, pH 10.5, and 100 μg of nonlabeled oleic acid as carrier were added, vortexed vigorously for 10 s, and centrifuged at 800 × *g* for 20 min at 21 °C. The upper 1 ml of the upper fraction was added to 10 ml of EcoLite liquid scintillant (ICN, Costa Mesa, CA) for scintillation counting.

For the NCEH assay, 1.17 mg of cholesteryl oleate, including 20×10^6 cpm of [^{14}C]cholesteryl oleate, plus a phospholipid mixture (71.4 μl, including phosphatidylcholine (15 mg/ml) and phosphatidylinositol (5 mg/ml), dissolved in chloroform) were placed in a 4-ml glass vial. Solvents were evaporated under a gentle stream of N_2. 2 ml of 0.1 M potassium phosphate, pH 7.5, was added, and after incubation at 37 °C for 10 min it was sonicated twice, each time for 1 min, separated by a 1-min interval. An additional 1.0 ml of 0.1 M potassium phosphate, pH 7.5, was added and incubated at 37 °C for 10 min. The mixture was sonicated for 4 × 30 s on ice with a 30-s interval between sonications. After sonication, 1.0 ml of 20% fatty acid-free BSA prepared in 0.1 M potassium phosphate (pH 7.5) was added. For each assay, 100 μl of substrate was mixed with 100 μl of sample and incubated for 30 min at 37 °C without shaking. Reaction termination, extraction, and scintillation counting were as above.

Subcellular Fractionation—Subcellular fractionation was performed as described (10).

Synthesis of Oleic Acid-linked Affi-Gel 102 Chromatography Resin—Oleic acid-linked Affi-Gel 102 resins were synthesized as described (11).

Preparation of Fat Tissue Sample for Chromatography—Following intracardiac catheterization, a perfusion of normal saline at 0.5 ml/min was performed for about 6 min. Fat was collected after perfusing overnight-fasted 5–6-month-old C57BL/6 mice. Samples were stored at −70 °C until use. WAT (10 g) was homogenized in 20 ml of homogenization buffer (0.25 M sucrose, 1 mM EDTA, 1 mM dithioerythritol, 20 μg/ml leupeptin, 2 μg/ml antipain, 1 μg/ml pepstatin, pH 7.5), and then centrifuged at 100,000 × *g* for 1 h at 4 °C. The infranatant and fat cake fractions were collected. The infranatant was concentrated to 7 ml in a Centricon Plus-20 apparatus (molecular weight cut-off 5000; Millipore Corp., Bedford, MA) using a membrane with 5000 a cut-off.

Fat Cake Protein Extraction—Lipase activity was quantitatively extracted from the fat cake fraction with acetone as follows. 10 ml of cold acetone were added to fat cake and incubated overnight at −20 °C. After centrifugation at 10,000 × *g* for 10 min at 4 °C, the upper aqueous phase was discarded, and 10 ml of chilled diethyl ether were added to the pellet. After incubation for 30 min at −20 °C, tubes were centrifuged at 10,000 × *g* for 10 min at 4 °C. The upper aqueous phase was discarded, and pellets were kept at room temperature for 10 min. After the addition of 10 ml of homogenization buffer, pellets were sonicated (setting 40; Artek Systems Corp., Farmingdale, NY) and then centrifuged at 10,000 × g at 4 °C for 10 min. The infranatant was collected. The residual fat cake was again sonicated. After centrifugation, all fractions of solubilized proteins in homogenization buffer were pooled. The fat cake extract was filtered through a 0.8-μm low protein binding membrane before application to an oleic acid-linked agarose column.

Oleic Acid-linked Agarose Chromatography—7 ml of concentrated infranatant or 8 ml of concentrated fat cake extract were applied to the gel (column HR 10/30; Amersham Biosciences) at a rate of 0.25 ml/min, followed by washing with buffer 1 (0.25 M sucrose in 10 mM phosphate buffer, pH 7.5) and buffer 2 (0.86 M NaCl in 10 mM phosphate buffer) at a flow rate of 1.0 ml/min. Bound proteins were eluted in a 0–1.0% linear gradient of CHAPS in 10 mM phosphate buffer, pH 7.5, with a 1.0 ml/min flow rate. Fraction volume was 4 ml. All steps were performed at 4 °C. Carboxylesterase activity was measured in each fraction, using PNPB as substrate.

Gel Permeation Chromatography—PNPB activity peaks were concentrated to 1 ml in a Centricon Plus-20 apparatus (molecular weight cut-off 5000) and then applied to a Superdex-200 fast protein liquid chromatography column (Amersham Biosciences). Proteins were eluted using 10 mM phosphate buffer (pH 7.5) containing 100 mM NaCl and 0.1% CHAPS. 1.0-ml fractions were collected, assayed for carboxylesterase and lipolytic activities, and analyzed by SDS-PAGE, followed by Coomassie Blue staining. Detectable bands were excised for MS analysis.

MS Identification—In-gel digestion of proteins was performed on the Investigator ProGest Robot (Genome Solutions, Ann Arbor, MI) as described (12). Samples of high protein abundance were analyzed by a liquid chromatography-MS system consisting of a nanoflow liquid chromatography system equipped with FAMOS Autosampler (LC Packings, Dionex, San Francisco, CA) and an LCQ Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). Peptides were separated by reversed phase high pressure liquid chromatography on a Picofrit column PFC7515-PP18-5 (New Objective, Woburn, MA). Buffer A was 95% water, 5% acetonitrile, and 0.1% formic acid. Buffer B was 90% acetonitrile, 10% water, and 0.1% formic acid. A flow rate 250 nl/min was used to separate the peptides. The column effluent was sprayed directly into the mass spectrometer. Low protein abundance samples were analyzed on an API QSTAR Pulsar Hybrid MS/MS mass spectrometer (Applied Biosystems/MDS SCIEX, Concord, Canada) equipped with a Nanospray Ion Source (Proxeon, Odense, Denmark) or on a Reflex IV matrix-assisted laser desorption ionization time-of-flight mass spec-

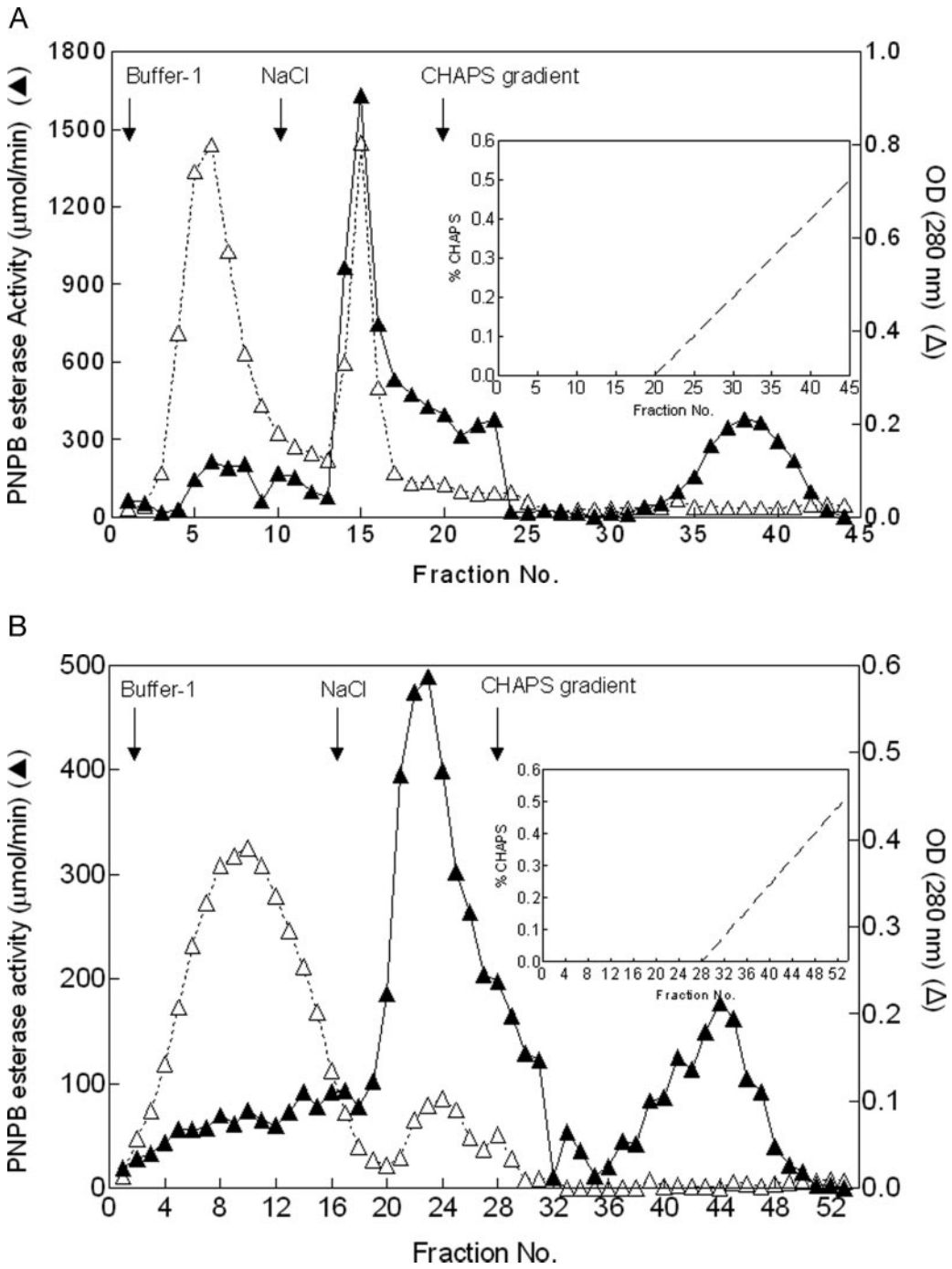

FIG. 1. **Oleic acid-agarose chromatograph of infranatant (*A*) and fat cake (*B*) showing total PNPB esterase activity (▲) and OD at 280 nm (△) of each fraction.** The CHAPS gradient is shown as a *dashed line* in the *inset*. *A*, in the infranatant, fractions 14–17 (corresponding to peak 1) and fractions 35–41 (peak 2) were pooled for further purification. *B*, in fat cake, fractions 20–27 (peak 1) and 39–47 (peak 2) were pooled.

trometer (Bruker Daltonics, Bremen, Germany) in the reflector mode. Prior to the analysis, samples were purified and concentrated on ZipTips (Millipore Corp., Billerica, MA). Spectra were searched against the NCBI (Bethesda, MD) data base using the Mascot software (Matrix Science, London, UK).

Protein Estimation—For chromatography, protein concentrations were estimated by absorption at 280 nm. Otherwise, proteins were quantified using the Bio-Rad protein assay kit with BSA as a standard.

Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gels 10% were run under reducing conditions (13) and stained with Coomassie Blue.

Western Blotting—Proteins separated by SDS-PAGE were electroblotted onto a polyvinylidene difluoride membrane in ice-cold 25 mM Tris, 192 mM glycine, buffer, pH 8.3, at 100 V for 1 h. Following transfer, the membrane was blocked with 5% skim milk and 0.1% Tween 20 in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) overnight at 4 °C and then incubated with a 1:3000 dilution of rabbit anti-TGH antibody (8). The membrane was washed three times for 10 min with TBS containing 0.1% Tween 20 and incubated for 1 h with a 1:10,000 dilution of peroxidase-conjugated goat anti-rabbit IgG in blocking buffer. After washing in similar condition, the bound antibody was detected by the ECL detection system (Roche Applied Science) as recommended by the manufacturer. Detection of the ER integral membrane marker protein, calnexin, was performed using a polyclonal rabbit anti-calnexin antibody (Stressgen, Victoria, Canada) in a 1:8000 dilution.

Differentiation of Fibroblasts—NIH 3T3-F442A fibroblasts were seeded onto 8-well Permanox slides (Nalge Nunc International, Naperville, IL) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Two days after the cells reached confluence, 0.25 μM dexamethasone, 0.5 mM isobutylmethylxanthine, and 3 μg/ml insulin were added to the culture medium. Cells were studied following differentiation, 6–8 days later.

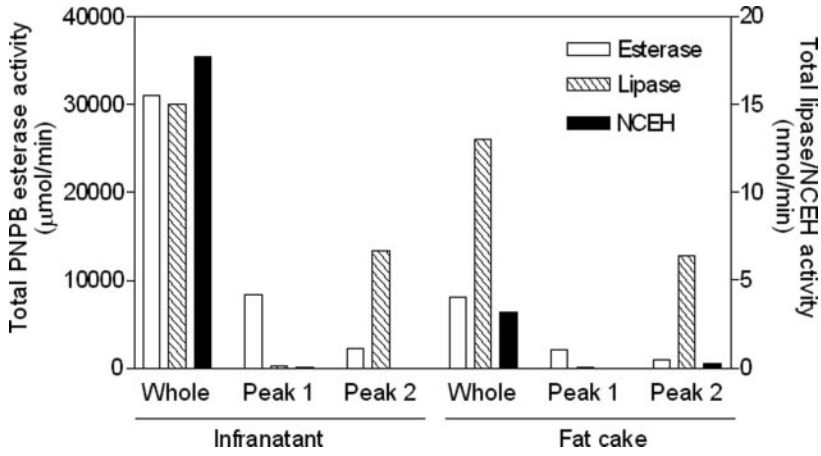

FIG. 2. **Recovery of PNPB esterase, lipase, and NCEH activities following oleic acid affinity chromatography of infranatant and fat cake fractions.**

Confocal Immunofluorescence Studies—Differentiated adipocytes were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 1% Triton X-100 in PBS for 2 min, and then probed with anti-perilipin (1:10,000) and anti-TGH (1:200) antibodies in the presence of 3% BSA. TGH was visualized by incubation with Alexa Fluor 488 donkey anti-rabbit IgG (1:500) and perilipin, with Alexa Fluor 594 goat anti-pig IgG (1:1000). All incubations were performed at 37 °C, and washes were at room temperature. After immunostaining, coverslips were mounted onto glass slides using ProLong Antifade kit. Images were captured with a Zeiss laser-scanning confocal imaging system.

RESULTS

Lipase and NCEH Activities in Subcellular Fractions of WAT—As shown in Table I, about 70% of total lipase activity was present in the cytosol, and about 10% was present in the fat cake fraction. These fractions are felt to correspond roughly to the coating of the lipid droplet (fat cake) and to the surrounding cytosol (infranatant), suggesting a potentially high biological importance in lipolysis. We therefore pursued lipase purification from these two fractions.

Purification and Identification of Carboxylesterases—Following oleic acid-agarose gel affinity chromatography, two peaks of PNPB esterase activity were observed both in infranatant (Fig. 1*A*) and fat cake fractions (Fig. 1*B*). In both fractions, the first peak was clearly distinct from the flow-through fraction, being retained on the column until elution under high salt concentrations (10 mM phosphate (pH 7.5) and 0.86 M NaCl). The second peak contained proteins strongly associated with the hydrophobic affinity resin and eluted by the CHAPS detergent gradient. Fig. 2 shows recovery of esterase and lipase activities from infranatant and fat cake extracts during the affinity purification. Lipase activity was almost exclusively confined to peak 2 of both fractions. Peaks 2 of fat cake and of infranatant also showed low but detectable cholesteryl esterase activity. For example, the ratio of lipase to NCEH activities was 26 in fat cake peak 2 and 162 in infranatant peak 2, in contrast to 1.6 in whole WAT homogenate (Table I). Therefore, most NCEH activity was lost during the purification under nonreducing conditions.

After SDS-PAGE analysis of fractions corresponding to peaks 1 and 2 of infranatant and of the fat cake extract, all protein bands detected with Coomassie Blue staining (Fig. 3) were excised for MS/MS analysis. In infranatant, MS/MS analysis of the 60-kDa peptide (Fig. 3) identified TGH (GI-16716505), based on on-line nano-liquid chromatography/MS/MS results. An example is shown in Fig. 4 for two of the MS/MS spectra obtained from the 60-kDa band. In both cases, the MS/MS spectra are dominated by cleavage of peptide bonds giving rise to y-type ions, which allows unambiguous peptide sequence determination. The other proteins detected by Coomassie Blue staining of infranatant peak 2 are listed in Fig. 3. Of note, by gel permeation chromatography, we found that the

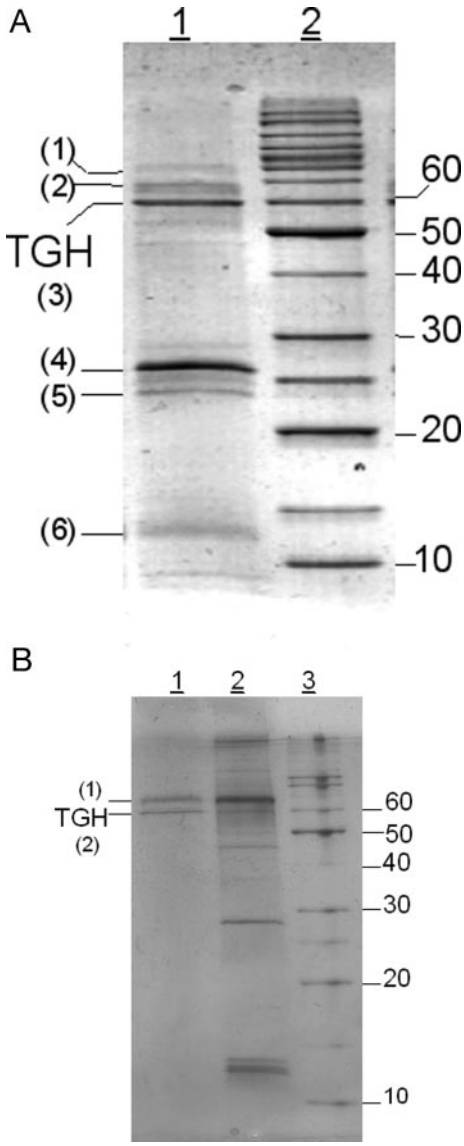

FIG. 3. *A*, SDS-polyacrylamide gel electrophoresis infranatant peak 2 (*lane 1*). *Lane 2*, molecular mass standards. To the *left* of *lane 1*, the positions of excised proteins are indicated. Proteins identified from the bands are as follows: cytokeratin 1 (*1*), albumin (*2*); albumin (*3*); TGH (*4*); cytokeratin 9 (*5*); apolipoprotein A-IV precursor (6), albumin (*7*), hemoglobin β-chain (*8*); apolipoprotein A-I (*9*), apolipoprotein A-I (*10*), and hemoglobin α-chain (fragment) (*11*). *B*, fat cake peak 2 (*lane 1*). *Lane 2*, whole fat cake extract. *Lane 3*, molecular mass standard. To the *left* of *lane 1*, the positions of excised proteins are indicated. Proteins identified from the bands are albumin (*1*) and TGH (*2*).

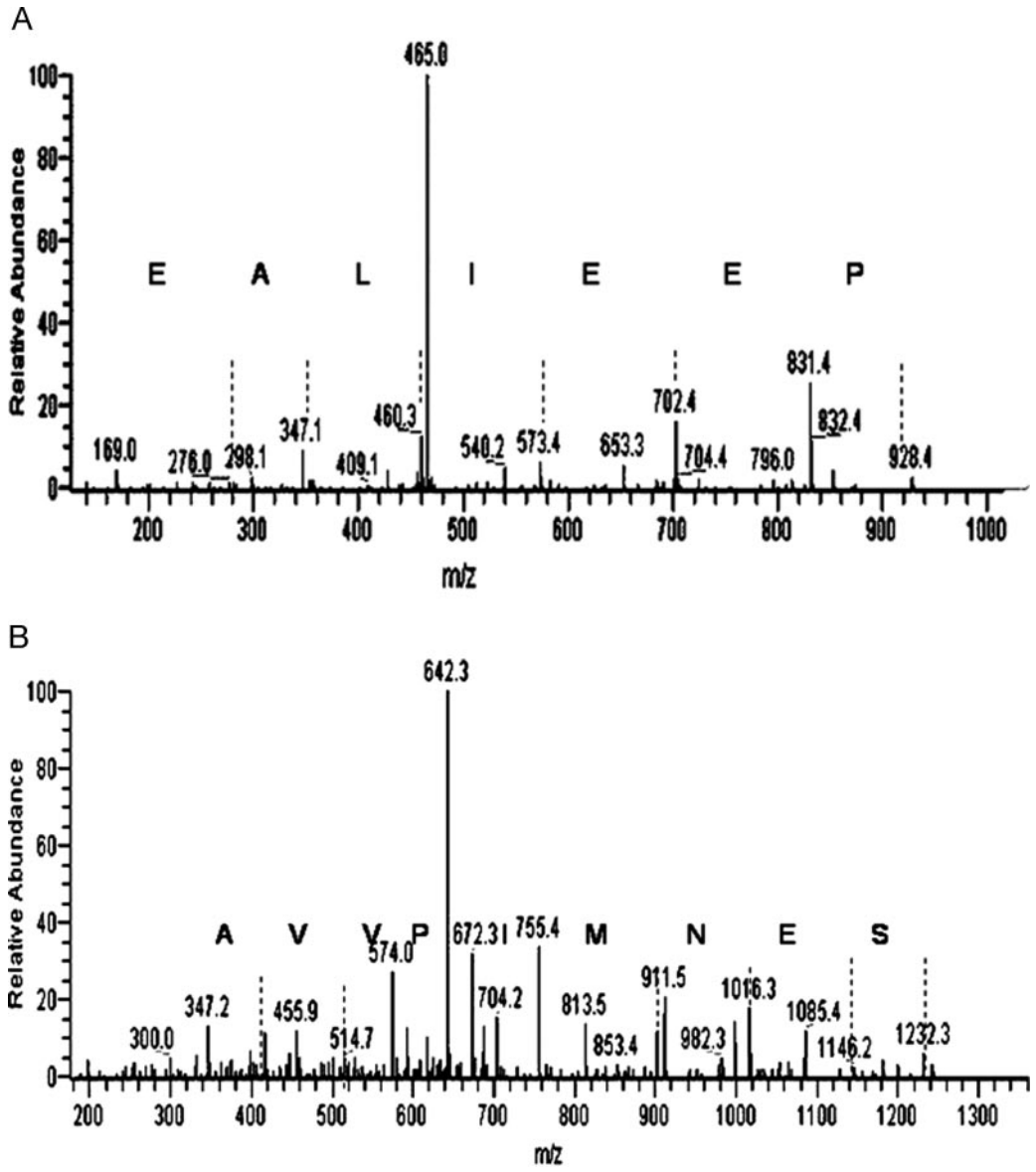

FIG. 4. **Examples of MS/MS spectra of peptides (y-series) produced from the 60-kDa peptide of infranatant peak 2, permitting its identification as TGH.** *A*, PEEILAE (TGH residues 331–337); *B*, SENMIPVVA (TGH residues 390–398).

esterase activity of infranatant peak 2 was confined to fractions in the 60–70-kDa molecular mass range (not shown).

Following SDS-PAGE of infranatant peak 1 (not shown), MS/MS analysis of 43 peptide fragments of an intensely Coomassie Blue-staining 80-kDa band identified esterase 1 (GI-6679689) unambiguously, with 54% sequence coverage. Infranatant peak 1 also contained a small amount of a 60-kDa peptides identified as TGH (not shown).

In fat cake peak 2, only two peptides were detectable by SDS-PAGE, at about 60 and 70 kDa (Fig. 3*B*). By MS/MS analysis, the 60-kDa peptide was revealed to be TGH (42 peptides, 21% sequence coverage). The 70-kDa peptide was albumin.

TGH Localization—By Western blotting of WAT subcellular fractions (Fig. 5), the distribution of TGH was compared with that of the ER membrane marker protein, calnexin, and the ER lumen marker protein, α-glucosidase II. TGH was present in microsomal and fat cake fractions. However, in contrast to calnexin and α-glucosidase II, a significant fraction of TGH was also present in the cytosol fraction.

By confocal microscopy, most TGH immunostaining was cytoplasmic. Some TGH colocalized with protein-disulfide isomerase in the ER lumen (not shown) and some with perilipin on the lipid droplet surface (Fig. 6).

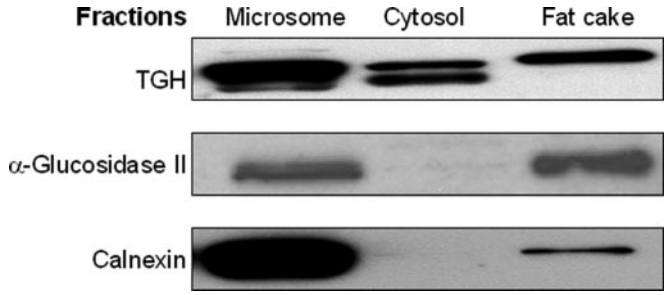

FIG. 5. **Western blotting for TGH, calnexin, and α-glucosidase II in WAT cell fractions.**

Lipolytic Activities and Lipase Peptide in HSL −/− Mouse WAT—Lipase activity in both infranatant and fat cake fractions (Fig. 7*a*) of WAT from 2-month-old HSL −/− mice was about 20% that of HSL +/+ controls. HSL −/− mice had no detectable NCEH activity (Fig. 7*b*) and no detectable immunoreactive HSL by Western blotting (not shown). In contrast,

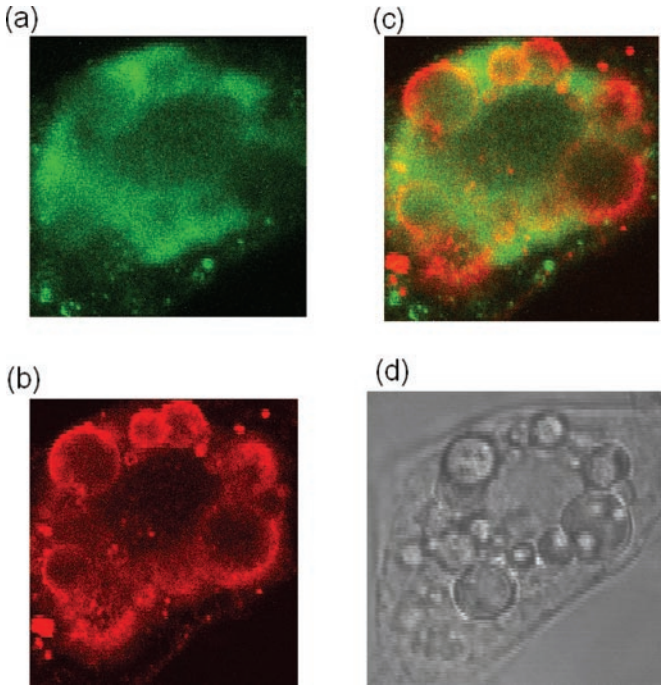

FIG. 6. **Immunolocalization of TGH in differentiated NIH 3T3-F442A fibroblasts.** *a*, TGH (*green*); *b*, perilipin (*red*); *c*, merged image; *d*, phase-contrast image.

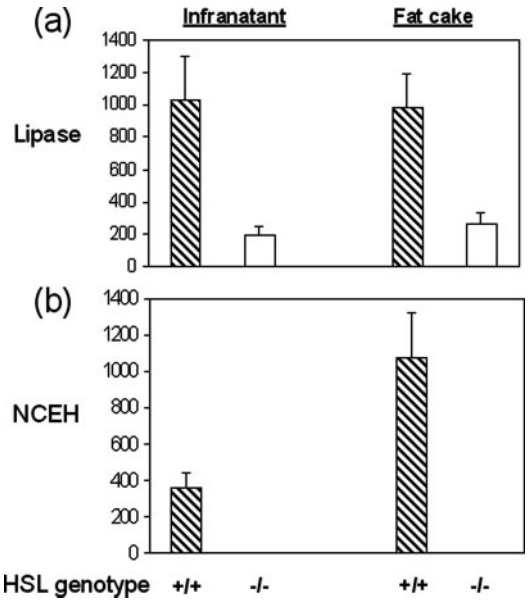

FIG. 7. **Activities of lipase (*a*) and NCEH (*b*) in infranatant and fat cake of HSL +/+ (*hatched bars*) and HSL −/− (open bars) WAT.** Enzyme activities are expressed as pmol/min/mg protein.

TGH peptide was easily detectable in both HSL +/+ and −/− WAT (not shown).

DISCUSSION

By functional proteomics, TGH (GI-16716505) was identified as a major lipase in WAT. In both infranatant and fat cake, the identity of TGH was established unambiguously based on the MS/MS sequencing of multiple peptides covering 52% of the TGH amino acid sequence in infranatant and 21% in fat cake. In both cases, we also observed a good correlation of experimentally determined M_r of the protein bands in question with that predicted from the TGH amino acid sequence.

In our preparation, other known WAT lipases were not detected, namely lipoprotein lipase (LPL), lysosomal acid lipase, and HSL. This was predictable. For LPL activity, the essential cofactor apolipoprotein CII will not copurify, and hence LPL activity is not detectable (14). Acid lipase requires acidic conditions for activity (pH optimal, 4) (15) and hence would be inactive under our assay conditions (pH 7.5). Of note, since LPL is extracellular and LAL is sequestered in lysosomes, neither is predicted to be of physiological importance for hydrolysis of lipid droplet TAGs.

HSL is physiologically important in adipocyte lipolysis but requires reducing conditions to retain activity. In Fig. 2, the whole infranatant and whole fat cake fractions were prepared under reducing conditions and presumably reflect the combined activities of HSL and of other intracellular neutral lipases. In contrast, peaks 1 and 2 were obtained by further purification under nonreducing conditions and are predicted to represent the activities of non-HSL esterases and lipases. Consistent with the concept that HSL is the main neutral cholesteryl esterase of WAT (6, 7), there is almost no detectable NCEH activity in peaks 1 and 2. However, peak 2, which binds strongly to the hydrophobic oleic acid-agarose column (Fig. 1), and in which TGH is concentrated, contains a substantial fraction of the total cellular lipase activity.

Therefore, a major fraction of *in vitro* lipolytic activity in WAT is attributable to TGH. Consistent with this, substantial lipase activity has been observed in homogenates of HSL −/− WAT (Fig. 7) (5, 6) and in HSL −/− mouse embryonic fibroblast following *in vitro* differentiation to adipocytes (7). To a first approximation, the level of lipase activity attributable to TGH may be sufficient to account for most or all non-HSL lipase activity in WAT. Conversely, however, our results cannot exclude the presence of non-HSL, non-TGH lipase(s) in WAT.

Although the esterase activity of peak 2 can be attributed to TGH, peak 1 does not contain sufficient TGH to explain its high esterase activity. The high level of esterase activity in peak 1 is probably due to esterase 1, a nonspecific, 60-kDa microsomal carboxylesterase reported to be highly expressed in liver and kidney (16). Carboxylesterases are present in a wide variety of organs and tissues of mammals and are implicated in the pharmacokinetics of drugs containing ester or amide bonds (17). Carboxylesterase activity has been reported in rat WAT (18, 19). To our knowledge, this is the first identification of esterase 1 in WAT. Its physiological significance in this tissue remains to be explored.

Total lipase activity (Fig. 2) is similar in both fat cake peak 2 (6.4 nmol/min) and infranatant peak 2 (6.7 nmol/min). These results indicate that TGH is the major non-HSL lipase in both fractions. In fat cake peak 2, only two peptide bands (Fig. 3*B*), were identified, TGH and albumin. In WAT infranatant peak 2, TGH is one of a small number of peptides (Fig. 3*A*). By gel permeation chromatography, the esterase activity in this peak is confined to the size range of TGH. Furthermore, none of the other identifiable peptides of infranatant peak 2 have known or predicted lipase or NCEH activities. Apolipoprotein A-I is a component of high density lipoprotein and is involved in cholesteryl ester transport. It might thus be expected to interact with CE or other hydrophobic molecules and to influence apparent TAG hydrolase or NCEH activities. However, the substrate concentrations for the NCEH and TAG hydrolase assays are several orders magnitude higher than those predicted for apolipoprotein A-I. Therefore, the observed levels of apolipoprotein A-I are not expected to markedly influence either assay. With this proviso, our results suggest that WAT TGH has both lipase and NCEH activities. These findings are consistent with previous publications in other tissues and other species, in which lipase (8) and NCEH (20) activities were reported for TGH.

In HSL −/− WAT homogenate, we detected about 20% of the lipase activity found in HSL +/+ WAT. As previously reported (5, 6), no detectable NCEH activity was present in −/− WAT. By Western blotting, TGH was easily identifiable in both HSL −/− and +/+ WAT. Given the low activity of TGH toward cholesteryl esters, the predicted level of TGH-related NCEH activity in whole WAT homogenate would be about 7 pmol/min/mg protein, which is undetectable with the assays used in crude homogenate. HSL −/− mice have been shown to have abnormally high amounts of cholesteryl esters in WAT (5). Thus, the small amount of NCEH activity provided by TGH is not in itself sufficient to normalize adipocyte cholesterol metabolism.

In vitro measurements do not necessarily indicate physiological activity. *In vivo*, lipolysis is determined both by intrinsic lipase capacity and by access of lipase(s) to TAGs of the lipid droplet. This has been elegantly demonstrated for HSL, which displaces from cytosol to the lipid droplet surface following β-adrenergic stimulation, a process that involves protein kinase A-mediated HSL phosphorylation (21). A reciprocal displacement is observed for perilipin A, a protein that coats the lipid droplet surface and that may form a barrier to access by lipases to their TAG substrate (22). Our work establishes that the level of TGH could potentially exert a substantial influence in lipolytic capacity in adipocytes. However, the true impact of adipocyte TGH on lipolysis cannot be deduced from this work. It will require studies by pharmacologic and/or genetic manipulation.

Both subcellular fractionation and confocal immunofluorescence microscopy suggest that TGH is present in the cytosol, in the ER, and also on the lipid droplet surface (Fig. 6). TGH has previously been isolated from pig liver microsomes (8) and from rat liver cytosol (20). In WAT, we compared the distribution of TGH with that of the ER luminal enzyme, α-glucosidase II. A greater fraction of TGH than of α-glucosidase II was present in the cytosol, suggesting that the presence of TGH in this compartment may not be due simply to spillage. Other possibilities cannot be eliminated, such as nonhomogeneous distribution of enzymes within adipocyte ER and selective disruption of TGH-enriched ER during preparation. Although we have no evidence to support this, it is possible that the cytosolic TGH may be due to microsomal breakage during preparation. Of note, the microsomal proteins TGH, calnexin, and α-glucosidase II were all present in fat cake. Part of this may be artifactual, due to nonspecific hydrophobic affinity of these proteins for the lipid droplet during subcellular fractionation.

However, on confocal microscopy, some TGH immunofluorescence is in immediate proximity to the lipid droplet surface, and some merges with that of perilipin, a marker of the lipid droplet surface (Fig. 6). These observations are consistent with a true *in vivo* relationship between the lipid droplet surface and ER. This has previously been suggested by electron microscopic data showing proximity of some ER cisternae with the lipid droplet surface (23) and by reports that lipid droplets may arise from the ER (24–26) and of the presence of other ER proteins on the lipid droplet surface (26, 27). Given that TGH, a microsomal protein, accounts for most non-HSL intracellular lipase activity in WAT at neutral pH *in vitro*, the possibility that ER may be directly involved in adipocyte lipolysis merits further study.

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Carboxylesterase 3 (EC 3.1.1.1) Is a Major Adipocyte Lipase

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