Subcellular localization, developmental expression and characterization of a liver triacylglycerol hydrolase

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The mechanism and enzymic activities responsible for the lipolysis of stored cytosolic triacylglycerol in liver and its re-esterification remain obscure. A candidate enzyme for lipolysis, a microsomal triacylglycerol hydrolase (TGH), was recently purified to homogeneity from pig liver and its kinetic properties were determined [Lehner and Verger (1997) Biochemistry **36**, 1861–1868]. We have characterized the enzyme with regard to its species distribution, subcellular localization, developmental expression and reaction with lipase inhibitors. The hydrolase co-sediments with endoplasmic reticulum elements and is associated with isolated liver fat droplets. Immunocytochemical studies localize TGH exclusively to liver cells surrounding capillaries. Both TGH mRNA and protein are expressed in rats during weaning. The enzyme covalently binds tetrahydrolipstatin, an inhibitor of lipases and of triacylglycerol hydrolysis. The enzyme is absent from liver-derived cell lines (HepG2 and McArdle RH7777) known to be impaired in very-low-density lipoprotein (VLDL) assembly and secretion. The localization and developmental expression of TGH are consistent with a proposed role in triacylglycerol hydrolysis and with the proposal that some of the resynthesized triacylglycerol is utilized for VLDL secretion.

Key words: endoplasmic reticulum, lipase, lipid droplet, very-low-density lipoprotein

INTRODUCTION

A large portion of newly made triacylglycerol (TG) is directed for storage in lipid droplets in the cytoplasm of liver. It has been demonstrated that a significant proportion of the very-low-density lipoprotein (VLDL) TG is derived from this storage pool and that TG present in this pool must undergo lipolysis followed by re-esterification to be assembled into TG-rich lipoproteins [1–5]. The unresolved issue is to what degree the stored TG is hydrolysed. It has been reported that in the intestinal mucosa the chylomicron TG arising from the 2-monoacylglycerol pathway (after feeding with oil) and those arising from the glycerol 3-phosphate pathway (after feeding with fatty acid methyl or ethyl ester) differed mainly in the composition of the fatty acids in the *sn*-2 position but showed remarkable similarity in the fatty acid compositions in the *sn*-1 and *sn*-3 positions [3]. This observation is consistent with the hydrolysis of TG made via the glycerol 3-phosphate pathway to 2-monoacylglycerol, which can then undergo re-acylation to TG via the 2-monoacylglycerol pathway. The situation of the provision of stored cytoplasmic TG for VLDL assembly in the liver is complicated by the fact that the adult liver apparently does not exhibit appreciable monoacylglycerol acyltransferase activity and hence it is unclear how the 2-monoacylglycerols would be re-esterified to TG. Experiments by Wiggins and Gibbons [2] indicated that in cultured primary rat hepatocytes stored TG might undergo complete lipolysis to glycerol and fatty acids. This view has been challenged more recently by results obtained from an analysis of the positional distribution of diacylglycerol moieties from VLDL and liver TG. The results pointed to great similarities in positional distribution and molecular association of the fatty acids between the *sn*-1,2-diacylglycerol moieties of the VLDL and liver TG, whereas the corresponding *sn*-2,3-diacylglycerols were distinctly different [4]. These differences could be accounted for by an only partial hydrolysis of stored TG (to diacylglycerol and perhaps to 2-monoacylglycerol) before re-esterification. It was also calculated that a minimum of 60% of the VLDL TG is derived from the stored TG by lipolysis/re-esterification [4], which is in agreement with the 70% contribution to this pool observed by a different group of investigators [2]. It is of interest to point out that a human hepatoma cell line, HepG2, which is used extensively in the studies of lipoprotein assembly and secretion, does not mobilize stored TG for lipoprotein assembly efficiently [6,7]. This could provide an explanation for why these cells assemble and secrete lipoprotein particles in the low-density lipoprotein but not the VLDL density range.

The nature of the lipase(s) that might be responsible for the hydrolysis of stored cytoplasmic TG is not known. A TG hydrolase (TGH) activity was recently purified to homogeneity from pig liver microsomes [8]. The enzyme hydrolyses long-chain TG *in vitro* to diacylglycerols and 2-monoacylglycerols and its activity can be inhibited by esterase/lipase inhibitors [8]. The present study shows that TGH is present in subcellular fractions in which lipoprotein assembly takes place. TGH is associated with the cytoplasmic TG droplet and the ontogeny of TGH seems to be co-ordinated with VLDL secretion by the liver. In addition, TGH is absent from cells that are impaired in VLDL assembly/secretion. These results suggest that TGH might be a candidate enzyme involved in the lipolysis of cytoplasmic TG.

MATERIALS AND METHODS

Materials

Diethyl-*p*-nitrophenyl phosphate (E600), 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK')-inactivated chymotrypsin from bovine pancreas, proteinase K, horseradish peroxidase conjugates of anti-(rabbit IgG), anti-(sheep IgG) and anti-(goat IgG), *p*-nitrophenyl palmitate and *p*-nitrophenyl laurate were

Abbreviations used: ER, endoplasmic reticulum; MAM, mitochondria-associated membranes; TG, triacylglycerol; TGH, triacylglycerol hydrolase; VLDL, very-low-density lipoprotein.

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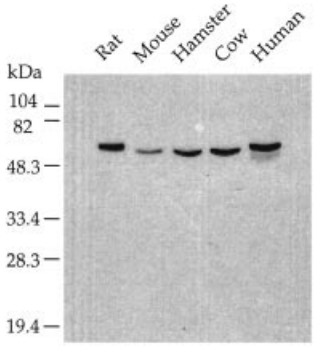

Figure 1 Species distribution of TGH

Total liver homogenate proteins (20 μg) from various species were subjected to SDS/PAGE [10% (w/v) gel], transferred to a nitrocellulose membrane and reacted with an affinity-purified anti-(pig TGH) antibody. The positions of molecular mass markers are shown at the left.

obtained from Sigma (St. Louis, MO, U.S.A.). ECL® Western blotting reagents were purchased from Amersham Canada (Oakville, Ontario, Canada). Triglyceride E kit was from Wako Chemicals (Richmond, VA, U.S.A.). Non-labelled and ^{14}C-labelled tetrahydrolipstatin were gifts from Dr. H. Lengsfeld (Hoffmann-LaRoche, Basel, Switzerland). Goat anti-(rat calreticulin) antibodies were kindly provided by Dr. M. Michalak (University of Alberta). Affinity-purified rabbit antibodies against the C-terminal 12 residues of rat phosphatidylethanolamine N-methyltransferase were generated in our laboratory [9]. Minimum essential medium, Dulbecco's modified Eagle's medium, sodium pyruvate, penicillin/streptomycin, fetal bovine serum and horse serum were from Gibco BRL (Life Technologies, Grand Island, NY, U.S.A.). A liver sample from biopsy of a 45-year-old woman was kindly provided with the patient's consent by Dr. V. G. Bain (University of Alberta, Edmonton, Alberta, Canada). All other chemicals and solvents were of reagent or better quality and were obtained from local suppliers.

Subcellular fractionations

Microsomes and crude mitochondria from 225–250 g Sprague–Dawley rats were prepared as described previously [10]. In brief, livers were washed with ice-cold Tris-buffered saline and homogenized with a Polytron (Brinkman Instruments, Ontario, Canada) for 30 s at low speed in 250 mM sucrose/25 mM Tris/HCl (pH 7.4)/5 mM EDTA to yield 20% (w/v) crude extract. Unbroken cells and cellular debris were removed by centrifugation for 10 min at 500 ***g***. Crude mitochondrial pellets were obtained by centrifugation of the 500 ***g*** supernatant at 15000 ***g*** for 10 min and microsomal membranes were prepared by centrifugation of the 15000 ***g*** supernatant for 1 h at 106000 ***g***. Fat droplets were prepared from floating fat at the top of the 106000 ***g*** supernatant by discontinuous gradient centrifugation as follows. The fat layer fraction (15 ml) containing contaminating cytosol was mixed with 15 ml of glycerol. The mixture was placed in a centrifuge tube, overlayed with 15 ml of a solution containing 250 mM sucrose, 50 mM Tris/HCl, pH 7.4 and 5 mM EDTA followed by 9 ml layer of 20 mM Tris/HCl (pH 7.4)/150 mM NaCl and centrifuged at 83000 ***g*** for 90 min (SW 28 rotor). Floating fat was removed and dispersed by brief sonication.

Mitochondria-associated membranes (MAM) and purified mitochondria were isolated by centrifugation of the crude mitochondrial fraction through a 30% (v/v) Percoll gradient [11]. Membranes enriched in rough endoplasmic reticulum (ER I), smooth ER (ER II) and Golgi-enriched membranes were prepared by a modified procedure [12] of Croze and Morré [13].

Immunoblotting

Proteins were separated by electrophoresis on 10% or 12% (w/v) polyacrylamide gels containing 0.1% SDS and transferred to a nitrocellulose membrane in ice-cold 25 mM Tris/HCl/192 mM glycine (pH 8.3)/20% (v/v) methanol for 1 h at 100 V constant voltage. After transfer, membranes were incubated for 1 h at room temperature or overnight at 4 °C with 5% (v/v) skimmed milk in T-TBS [20 mM Tris/HCl (pH 7.4)/150 mM NaCl/0.1% (v/v) Tween 20] and then incubated for 1 h with 0.3 μg/ml anti-(pig TGH) polyclonal antibody (affinity-purified on a CNBr column containing covalently linked TGH), with 0.5 μg/ml anti-(rat phosphatidylethanolamine N-methyltransferase) or anti-(rat calreticulin) polyclonal antibody (1:500 dilution) in T-TBS containing 1% (v/v) milk. After being washed with T-TBS containing 5% (w/v) milk, membranes were incubated for 1 h with a 1:20000 dilution of peroxidase-conjugated goat anti-(rabbit IgG) for TGH and phosphatidylethanolamine N-methyltransferase or rabbit anti-(goat IgG) for calreticulin in T-TBS containing 1% (v/v) milk. Membranes were extensively washed with T-TBS and the bound antibody was detected by enhanced chemiluminescence in accordance with instructions from the manufacturer.

Northern blotting

mRNA species (3 μg) isolated from liver samples by a Quick Prep™ kit (Pharmacia) were subjected to electrophoresis on a 1.2% (w/v) agarose/formaldehyde gel and transferred to HyBond-N$^+$ membrane. The TGH mRNA was detected by incubation of the membrane with ^{32}P-labelled cDNA for rat TGH followed by autoradiography.

Immunocytochemical localization

Liver samples were fixed in paraformaldehyde immediately after surgical removal, embedded in paraffin, cut into sections of 4 μm thickness and placed on glass slides coated with 3-aminopropyltriethoxysilane. After deparaffination and rehydration, the sections were incubated in 10% (v/v) H_2O_2 in methanol to quench endogenous peroxidase activity. The samples were washed with PBS and non-specific binding sites were blocked by incubation with 10% (v/v) goat serum. The sections were then incubated with affinity-purified anti-TGH polyclonal antibody. The antibody-specific staining was detected with Vectastain ABC reagent (Vector Laboratories, Burlingame, CA, U.S.A.) as directed by the manufacturer. To minimize variations in staining, sections collected at different times were placed and processed on the same slide. Cell nuclei were counterstained with haematoxylin.

Lipid mass analysis

Lipids were extracted from aliquots of subcellular fractions by chloroform/methanol (2:1, v/v) by the method of Folch et al. [14]. The phospholipid content of the lipid extracts was determined by the method of Chalvardjian and Rudnicki [15]. TG mass was determined by using a Triglyceride E kit with trioleoylglycerol as standard.

Cell culture

Hepatocytes were isolated from male Sprague–Dawley rats (body weight 125–150 g) fed *ad libitum*, by perfusion of the liver with collagenase. The cells were cultured in Dulbecco's modified Eagle's medium containing 15% (v/v) fetal bovine serum [16]. HepG2 cells, obtained from ATCC, were cultured in minimal Eagle's medium containing 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 i.u./ml penicillin, 100 μg/ml streptomycin and 10% (v/v) fetal bovine serum. McArdle RH7777 cells, obtained from ATCC, were cultured in Dulbecco's modified Eagle's medium containing pyruvate, antibiotics, 10% (v/v) fetal bovine serum and 10% (v/v) horse serum. All cutures were maintained in 100 mm dishes (Corning) at 37 °C in humidified air/CO_2 (19:1) (89–91%-satd. with water vapour).

Partial hepatectomy

Rats were anaesthetized with ether and subjected to midventral laparotomy and subsequent removal of two-thirds of the liver, by the method of Higgins and Anderson [17]. On sham-operated rats, laparotomy, exposure and manipulation of the liver were performed without excision. Animals were killed at various intervals after operation, livers were removed, washed in ice-cold saline and homogenates were prepared with a Polytron.

Labelling of TGH with tetrahydrolipstatin

Purified TGH (10 μg) was incubated for 1 h at room temperature in 100 mM Tris/HCl, pH 8.0, in the presence of one of the following: 1 mM E600, 5 mM $HgCl_2$, 0.1 μg of chymotrypsin or proteinase K in a final volume of 20 μl. A 15 μl portion was removed at the end of the incubation and assayed for lipase activity; to the rest was added 500000 d.p.m. of [^{14}C]tetrahydrolipstatin in 2 μl of acetone and the incubation was continued for 1 h at room temperature. The reaction was stopped by the addition of electrophoresis sample buffer containing SDS and 2-mercaptoethanol. The samples were boiled and subjected to electrophoresis on denaturing 12% (w/v) polyacrylamide gels. Gels were stained with Coomassie Blue and destained in 5% (v/v) methanol and 7.5% (v/v) acetic acid. Gels were incubated for 10 min in acetic acid, soaked for 90 min in 20% (w/v) 2,5-diphenyloxazole in acetic acid, washed for 30 min in water, dried under vacuum and exposed to Kodak X-Omat X-ray film at −70 °C for 4 days [18].

Other methods

Protein concentration was determined by using the Bio-Rad Protein Assay kit with BSA as a protein standard. Some SDS/polyacrylamide gels were silver-stained by the procedure of Rabilloud et al. [19].

RESULTS

Species distribution

TGH distribution in various mammalian species was analysed by immunoblotting with affinity-purified polyclonal antibody raised against pig liver TGH. Figure 1 shows that the immunoreactive protein has wide species distribution, being present in livers from human, cow, rat, mouse and hamster.

Subcellular localization

Subcellular fractionations of a rat liver homogenate on sucrose and Percoll gradients revealed that TGH was associated in approximately the same amounts (same amount of protein loaded) in the various ER elements, including the rough ER (ER I), the smooth ER (ER II) and the MAM, whereas little of the enzyme co-sedimented with enriched mitochondrial fractions (Figure 2). The specific activity of an ER marker NADPH:cytochrome-*c* reductase in MAM is typically approx. 25% of that in the ER II (a less dense ER fraction enriched in smooth ER) and 46% of that in ER I (a more dense ER fraction enriched in rough ER). MAM has been shown to be enriched with respect to microsomes in its specific activity of phosphatidylserine synthase and contains about the same specific activities of choline or ethanolamine phosphotransferases and phosphatidylethanolamine N-methyltransferase as in microsomes [11]. Es-

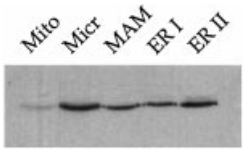

Figure 2 Subcellular localization of TGH

Membrane protein (10 μg) isolated from rat liver was separated by SDS/PAGE [12% (w/v) gel] and transferred to a nitrocellulose membrane; the presence of TGH was detected by immunoblotting. Abbreviations: Mito, crude mitochondria; Micr, microsomes; ER I, rough ER; ER II, smooth ER.

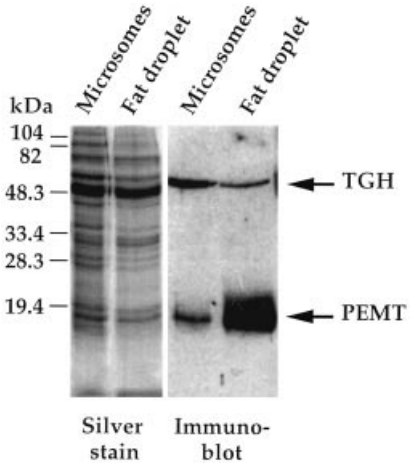

Figure 3 Protein composition of microsomes and isolated fat droplets from rat liver

Rat liver microsomal and fat droplet protein (4 μg) was separated by SDS/PAGE [12% (w/v) gel] and either silver-stained or transferred to a nitrocellulose membrane and reacted with anti-(pig TGH) and anti-[rat phosphatidylethanolamine N-methyltransferase (PEMT)] antibodies. The positions of molecular mass standards are indicated at the left.

Table 1 Lipid composition of isolated fat droplets

Total lipid extracts from rat liver microsomes and fat droplet fractions were prepared and analysed for phospholipid (PL) and TG content. Results are means ± S.D. for three experiments

Fraction	TG (μmol/mg of protein)	PL (μmol/mg of protein)	TG-to-PL ratio
Microsomes	0.06 ± 0.01	0.7 ± 0.1	0.09
Fat droplets	38.0 ± 5.0	3.1 ± 0.2	12.1

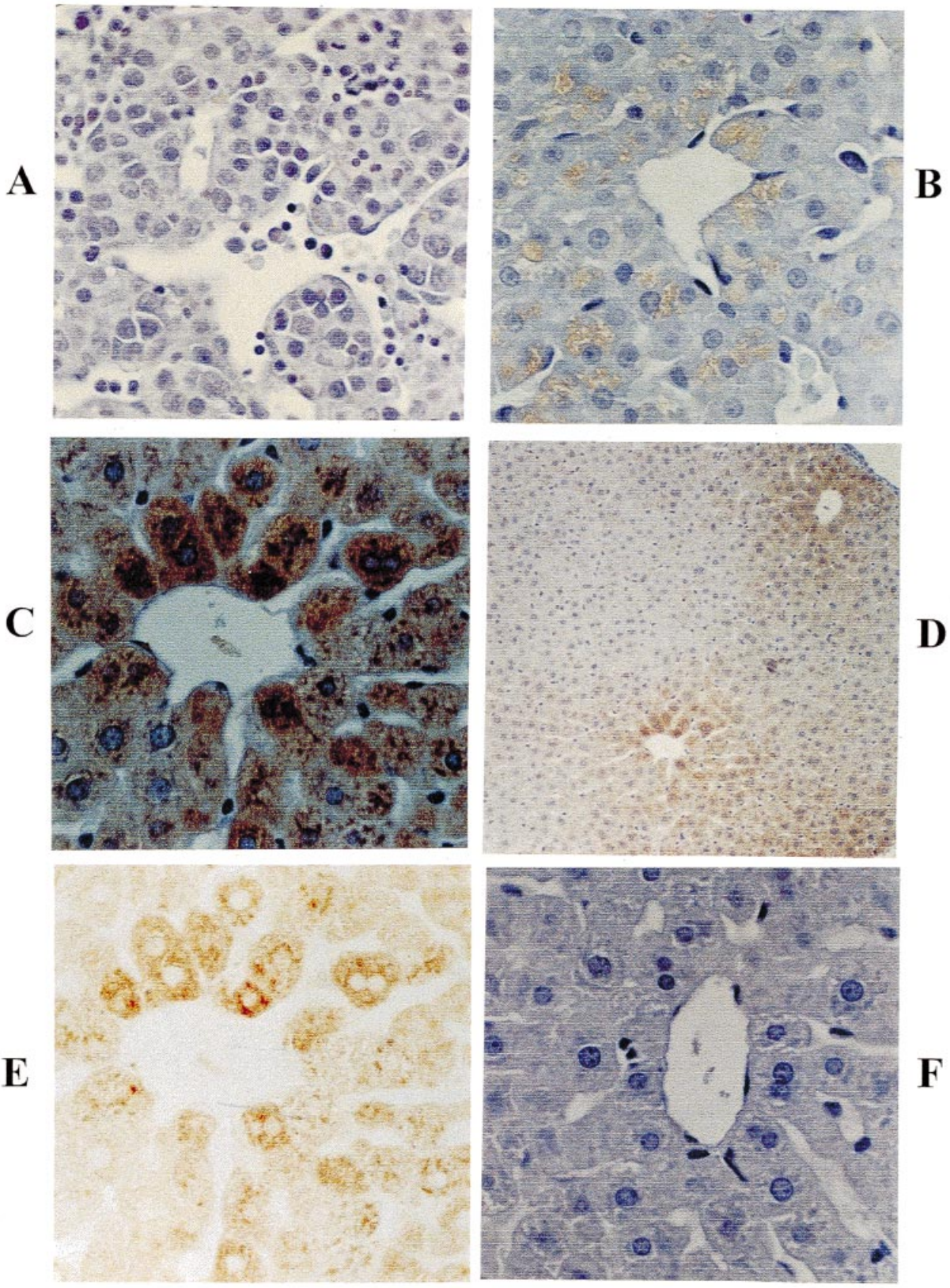

Figure 4 Immunocytolocalization of rat liver TGH

Liver sections from newborn (**A**), 4-day-old (**B**) and 31-day-old (**C**–**F**) rats were fixed in paraformaldehyde and embedded in paraffin and the immunoreactivity of anti-TGH antibody (**A**–**E**) (brown staining) was localized with a biotin–avidin detection system. (**F**) Incubation of adult liver section with the secondary antibody alone. (**A**–**C**, **E** and **F**) were taken with a ×40 objective lens with a ×10 eyepiece; (**D**) was taken with a ×10 objective lens with a ×10 eyepiece. Nuclei were counterstained with haematoxylin (blue).

sentially no immunoreactivity for TGH was found in fractions enriched in the Golgi membranes (results not shown). TGH activity in the organelles was correlated with the observed immunoreactivity.

Isolation and characterization of fat droplets

The TGH was also found to be localized on what seemed to be a special segment of ER in close contact with the intracellular lipid storage droplets (Figure 3). The lipid droplet fraction was obtained by discontinuous glycerol- and sucrose-density-gradient centrifugation. Lipid analysis showed that these droplets had a very high lipid content as compared with microsomes and were highly enriched in TG with a molar ratio of TG to phospholipid of 12:1 (Table 1). In comparison, the ratio of the two lipid classes in microsomal membranes was 0.09 (Table 1). SDS/PAGE analysis of the fat layer fraction showed a similar protein profile to that of microsomal membranes (Figure 3). Corresponding immunoblotting of these fractions indicated not only the presence of TGH but also a remarkable enrichment in an integral membrane protein phosphatidylethanolamine N-methyltransferase 2 (Figure 3) that had previously been shown to co-localize with liver microsomes and the MAM [9,20].

Immunocytochemical localization and developmental expression of TGH

Staining of liver slices with affinity-purified antibody raised against pig TGH showed that the expression of TGH was developmentally regulated (Figure 4). Little or no staining was observed in livers isolated from newborn rats (day 0; Figure 4A) or 4-day-old animals (Figure 4B), whereas intense staining was observed in livers from 31-day-old rats (Figures 4C–4E). The enzyme is expressed almost exclusively in cells adjacent to capillaries leading to the central vein (Figures 4C and 4D). TGH seems to be localized throughout the cytoplasmic region in a web-like fashion characteristic of ER and is absent from the nucleus (Figure 4E). In a control experiment, staining of adult liver tissue with secondary antibody alone did not produce any horseradish peroxidase cross-reactivity, indicating the specificity of the anti-TGH antibody reaction (Figure 4F). The expression of the rat liver TGH gene seems to be temporally regulated. The mRNA for TGH and, correspondingly, the enzyme were not detected during the early stages of development but expression seems to coincide with the period of weaning, because the enzyme and TGH mRNA became detectable after day 11 (Figures 5A and 5B). A more detailed analysis showed a gradual increase in expression of the enzyme between days 11 and 21 after birth (Figure 5C). Immunoblotting of the resident ER protein calreticulin was used as a control for the amount of protein loaded on the gel.

Partial hepatectomy

The activities of some extracellular TG lipases, such as hepatic and lipoprotein lipases, have been shown to be altered during liver growth after partial hepatectomy [21,22]. However, the levels of TGH protein were not altered in regenerating livers (results not shown).

Covalent modification of TGH with tetrahydrolipstatin

Tetrahydrolipstatin has been shown to block the activity of acylglycerol lipases by interacting with the nucleophilic serine residue in the active site [23]. Moreover, tetrahydrolipstatin decreases the secretion of VLDL TG by 80% from primary

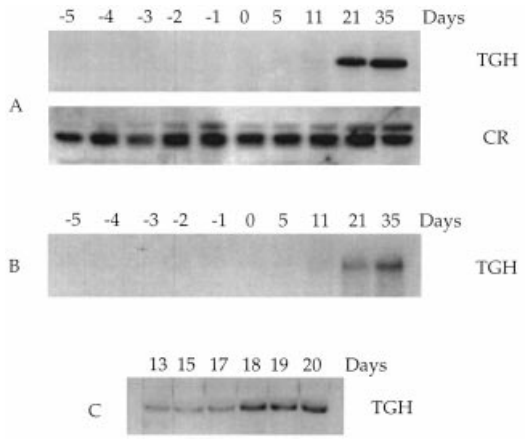

Figure 5 Developmental expression of rat liver TGH

(**A**) Proteins of liver homogenates (25 μg) prepared from rats of different ages were resolved by SDS/PAGE [12% (w/v) gel], electroblotted to a nitrocellulose membrane and reacted with anti-(pig TGH) antibody. Membranes were stripped and reprobed with an antibody raised against an ER-resident protein, calreticulin (CR). (**B**) mRNA (3 μg) extracted from livers of rats of the indicated ages was separated on a 1.2% (w/v) agarose/formaldehyde gel, transferred to a HyBond-N^+ nylon membrane and probed with ^{32}P-labelled TGH cDNA. The mRNA for protein disulphide isomerase mRNA was used as a control for the amount of mRNA loading; the result has been published elsewhere [45]. (**C**) Immunoblot analysis of 10 μg of protein from liver homogenates prepared from 13–20-day-old rats with anti-(pig TGH) antibody.

hepatocytes [24]. We therefore examined whether TGH would react with tetrahydrolipstatin. Incubation of purified TGH with radiolabelled tetrahydrolipstatin resulted in a covalent modification of the enzyme (Figure 6). Preincubation with $HgCl_2$, which has been shown to inhibit TGH activity [8], did not alter the level of labelling, indicating that the $HgCl_2$ inhibitory effect is due to a mechanism that does not prevent the access of

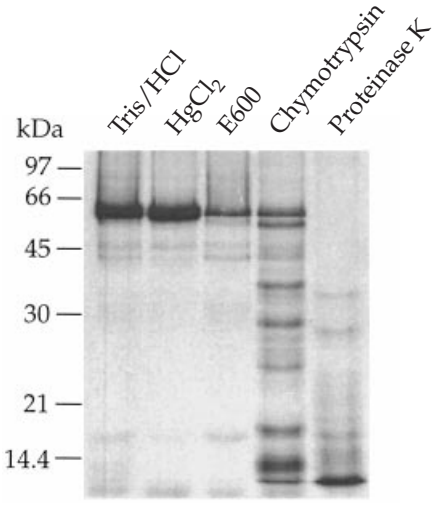

Figure 6 Covalent modification of TGH with tetrahydrolipstatin

Purified TGH (10 μg) was incubated for 1 h with Tris/HCl, with the TGH inhibitors (E600 or $HgCl_2$) or with proteases (chymotrypsin or proteinase K) before the addition of [^{14}C]tetrahydrolipstatin. After a 1 h incubation period with the radiolabelled inhibitor, the reaction was stopped by the addition of electrophoresis sample buffer. The samples were boiled and resolved by SDS/PAGE [12% (w/v) gel]; the interaction of tetrahydrolipstatin with TGH was analysed by fluorography.

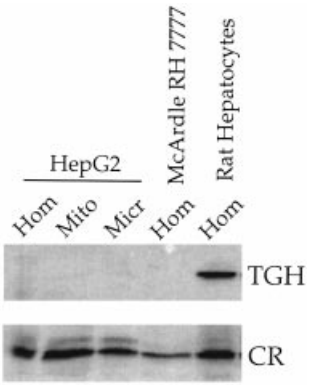

Figure 7 Absence of TGH from hepatoma cells

Total homogenate or organelle proteins (20 μg) isolated from hepatoma cells or primary hepatocytes were separated by SDS/PAGE [10% (w/v) gel], transferred to a nitrocellulose membrane and reacted with anti-(pig TGH) antibody. Membrane was stripped and reprobed with anti-[rat calreticulin (CR)] antibody. Abbreviations: Hom, total homogenate; Mito, crude mitochondria; Micr, microsomes.

tetrahydrolipstatin to the active-site serine residue. In contrast, preincubation with E600 (diethyl-*p*-nitrophenyl phosphate), an inhibitor known to modify the active-site serine residue of lipases [25,26], diminished the labelling of TGH with tetrahydrolipstatin. Protease-treated enzyme retained the ability to bind tetrahydrolipstatin (Figure 6); the active-site serine residue therefore retains its nucleophilicity. These results suggest that despite the extensive proteolytic degradation, the conformation of active site of the enzyme seems to remain relatively unchanged.

Absence of TGH from McArdle RH7777 and HepG2 hepatoma cell lines

Transformed cell lines McArdle RH7777 (rat hepatoma) and HepG2 (human hepatoma) are frequently used as models for the study of lipoprotein assembly and secretion by the liver, although production in these cells seems to be deficient in several aspects when compared with cultured primary hepatocytes. Immunoblot analysis of cell homogenates and subcellular fractions showed that TGH was absent from the hepatoma cells, whereas cultured rat hepatocytes retained the enzyme (Figure 7).

DISCUSSION

One of us recently reported [8] the purification and partial characterization of a microsomal TGH that was postulated to be a candidate enzyme responsible for the lipolysis of cytoplasmic stores of TG. TGH is expressed in the liver, intestine and kidney but is absent from heart [8]. The enzyme seems to have a wide species distribution. Antibody against the pig TGH cross-reacts with proteins in the livers from rat, mouse, hamster, cow and human. The corresponding rat and mouse liver microsomal hydrolases were purified in accordance with the protocol [8] used for purification of the pig enzyme, which indicates that the rodent hydrolases have similar physical properties to those of the pig enzyme. It therefore seems that TGH is very similar in the five mammalian species examined.

TGH is localized to ER elements and intracellular lipid droplets

Immunoblot analysis of rat liver subcellular fractions prepared by sucrose- and Percoll-density-gradient ultracentrifugation showed that the hydrolase co-sediments with smooth and rough ER and with an ER-like membrane fraction, MAM. The latter has been found to be enriched as compared with the bulk of the ER in lipogenic enzymes such as phosphatidylserine synthase, diacylglycerol acyltransferase and acyl-CoA:cholesterol acyltransferase [11], as well as the liver-specific enzyme phosphatidylethanolamine N-methyltransferase 2, which catalyses the conversion of phosphatidylethanolamine to phosphatidylcholine [9]. In addition, microsomal TG transfer protein and apolipoprotein B-containing lipoproteins are present in the lumen of the MAM [27]. These results are indicative of not only a possible role for this region of the ER in supplying lipids for nascent VLDL but also the active participation of MAM in lipoprotein particle assembly. Rusiñol et al. [27] also suggested that the MAM might be involved in the formation of the cytoplasmic TG storage pool on the basis of the enrichment of diacylglycerol acyltransferase activity and other lipid biosynthetic enzyme activities that might supply lipids for the surface layer of the lipid droplet. The localization of TGH to MAM indicates a possible role for this membrane in TG mobilization.

Because TGH is proposed to be involved in mobilization of stored TG, it makes sense that TGH should interact with lipid droplets in a manner analogous to that of the adipose tissue hormone-sensitive lipase [28]. We have prepared a TG-rich (fat droplet) fraction from rat liver and showed that the protein profile of the fat droplet was similar to that of microsomal membranes, suggesting that the lipid droplet is associated with the ER. In addition, immunoblot analysis clearly demonstrated the presence of TGH in the lipid droplet as well as phosphatidylethanolamine N-methyltransferase 2. Because this methyltransferase is exclusively localized in the MAM [9] and was strikingly enriched in the droplets, perhaps an area of continuity might exist between the surface of the lipid droplet and MAM and/or ER. Freeze-fracture electron microscopy of adipocyte lipid droplets showed continuity with an ER-like membrane [29]. A lipid droplet that is MAM- or ER-associated rather than in the cytosol would obviate the necessity for specialized lipid transfer proteins to mediate a vectorial transfer of released fatty acids and partial acylglycerols from the lipid droplet to the ER elements in which TG synthesis occurs.

TGH expression is confined to centrilobular region of parenchymal cells and is developmentally regulated

Immunocytochemical localization studies showed that, in adult rat liver, TGH is localized around vessels leading to central (terminal hepatic) venules, the site at which one would expect active lipoprotein synthesis and secretion. The antibody staining is visible only in cytoplasmic regions of liver parenchymal cells and is absent from endothelial cells.

The temporal regulation of the synthesis of TGH mRNA and protein coincides with weaning and the ontogeny of VLDL TG secretion by rat liver [30]. The expression of TGH was undetectable in fetal livers and very low in livers isolated from 4-day-old and 11-day-old rats. TGH expression seems to increase gradually during weaning, a phenomenon that might be related to an increased intake of dietary fat composed of TG with long-chain fatty acids rather than TG with medium-chain fatty acids found in milk. During the suckling period, the high-fat (from milk), low-carbohydrate nature of the diet is associated with high rates of fatty acid oxidation and gluconeogenesis. At the time of weaning, gluconeogenesis declines and the liver switches to the adult mode of metabolism with carbohydrate abundance. These changes are associated with increases in fatty acid synthesis *de*

novo, an increase in TG storage as compared with the suckling animals [30] and VLDL TG output.

Adult hepatocytes undergo de-differentiation after partial hepatectomy and acquire fetal and neonatal features [31,32]. Partial hepatectomy did not seem to change the expression of TGH. Previous studies have shown that stressful situations (such as surgery) produce a decrease in plasma insulin levels and increase the levels of catecholamines [33], glucagon and glucocorticoids [34], although catecholamine levels are usually higher in hepatectomized than in sham-operated animals [34]. Glucocorticoid and insulin levels affect TG synthesis and VLDL secretion [35]. However, our preliminary results suggest that TGH expression is not subject to regulation by glucocorticoids or insulin. The lack of apparent regulation of TGH expression and activity during stress lends support to a role for TGH in the lipolysis of stored TG because it has been demonstrated that lipolysis of the hepatic TG storage pool was not affected by either insulin or glucagon [2].

TGH is covalently modified by a lipase inhibitor, tetrahydrolipstatin

Tetrahydrolipstatin inactivates lipases by forming a covalent bond with the active-site serine residue [23]. Incubation of primary rat hepatocytes with tetrahydrolipstatin decreases the secretion of VLDL TG by 80%, suggesting that tetrahydrolipstatin might impair the mobilization of the intracellular TG storage pool via lipolysis [24]. Our results show that TGH is subject to modification by tetrahydrolipstatin *in vitro* and is therefore a likely target of tetrahydrolipstatin action *in vivo*.

TGH and VLDL assembly

The assembly of VLDL in the liver requires the synthesis of neutral lipid, phospholipid and apolipoprotein B. Although the requirement for phosphatidylcholine synthesized *de novo* in lipoprotein assembly has been clearly established [16,36–39], less is known about the origin of VLDL TG. A simple model of direct transfer of TG synthesized *de novo* to a nascent apolipoprotein B-containing particle has been compromised by studies in primary hepatocytes demonstrating that TG synthesized from extracellular fatty acids is partly utilized for VLDL assembly and is also stored within the cell [40,41]. Approximately 70% of VLDL TG is derived from intracellular TG storage pools, whereas only 30% is derived from TG synthesized *de novo* [1–5]. The stored TG does not seem to be transferred directly to the developing lipoprotein particle; instead, the results are consistent with lipolysis of the TG to partial glycerides followed by re-esterification [2–4,42–44]. The intracellular location of the lipolysis/re-esterification cycle was not known; neither were the identities of the lipase and acyltransferases involved. Initially a lysosomal acid lipase was suggested to be involved in the process [1]. However, TG mobilization is not inhibited by chloroquine, an inhibitor of lysosomal hydrolases [2]. Moreover, treatment with insulin did not affect the lipolysis, suggesting that hormone-sensitive lipase was not involved. These observations pointed to the existence of another intracellular lipase that is involved in the lipolysis of stored TG.

The studies reported here suggest that TGH is involved in the mobilization of TG for VLDL secretion. We have generated McArdle RH7777 cells stably expressing rat TGH cDNA and obtained evidence that these cells show increased mobilization (lipolysis) of stored TG, some of which is utilized for lipoprotein assembly (R. Lehner and D. E. Vance, unpublished work).

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